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Basement Membrane Complexes with Biological Activity

Hynda K. Kleinman,* Mary L. McGarvey, John R. Hassell, Vicki L. Star, Frances B. Cannon, Gordon W. Laurie, and George R. Martin

Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: We have studied the reconstitution of basement membrane molecules from extracts prepared from the basement membrane of the EHS tumor. Under physiological conditions and in the presence of added type IV collagen and heparan sulfate proteoglycan, gellike structures form whose ultrastructure appears as interconnected thin sheets resembling the lamina densa zone of basement membrane. The major components of the reconstituted structures include laminin, type IV collagen, heparan sulfate proteoglycan, entactin, and nidogen. These components polymerize in constant proportions on reconstitution, suggesting that they interact in defined proportions. Molecular sieve studies on the soluble extract demonstrate that laminin, entactin, and nidogen are associated in large but dissociable complexes which may be a necessary intermediate in the deposition of basement membrane. The reconstituted matrix was biologically active and stimulated the growth and differentiation of certain cells.

Connective tissues contain different species of collagens, glycoproteins, and proteoglycans (Eyre, 1980; Bornstein & Sage, 1980; Kleinman et al., 1981; Hay, 1982). These macromolecules form the matrix structures that contribute to the physical characteristics of tissues as well as provide unique substrates for the resident cells. For example, the matrix produced by cultured fibroblasts consists of a dissociable complex of type I collagen, fibronectin, and heparan-containing and chondroitin sulfate containing proteoglycans (Hedman et al., 1983). Similarly, Schubert & LaCorbiere (1980) demonstrated the presence of complexes containing collagen, proteoglycan, and glycoprotein in the media of cultured myoblasts and neural retinal cells. These complexes which they termed adherons also supported the attachment of cells (Schubert & LaCorbiere, 1982; Schubert et al., 1983).

In the present paper, we examine the macromolecular complexes involved in the formation of basement membranes. Basement membranes are thin but continuous sheets that separate epithelium from stroma and surround nerves, muscle fibers, smooth muscle cells, and fat cells (Kefalides, 1973; Vracko, 1974; Timpl & Martin, 1982; Laurie et al., 1983). Basement membranes contain type IV collagen (Kefalides, 1973; Orkin et al., 1976), the glycoproteins laminin (Timpl et al., 1979; Chung et al., 1979), entactin (Carlin et al., 1981), and nidogen (Timpl et al., 1983), and heparan sulfate proteoglycans (Kanwar & Farquhar, 1979; Hassell et al., 1980, 1985). In various studies, these materials show a codistribution (Leivo et al., 1982; Hayman et al., 1982; Laurie et al., 1982, 1984b) both within the lamina densa and within its extensions

across the lamina lucida. Using electron microscopy, the components appear as a network of 5-nm-wide cords (Laurie et al., 1984), and by using electron microscopy, their codistribution suggests that the formation of basement membrane occurs through their interactions. Type IV collagen molecules form intermolecular disulfide bonds and associate in a continuous network (Timpl et al., 1981; Veis & Schwartz, 1981; Fessler & Fessler, 1982; Bächinger et al., 1982; Yurchenko & Furthmayr, 1984) which can be visualized in basement membranes digested with plasmin (Inoue et al., 1983).

Various components of the basement membrane are known to interact with each other. In vitro studies with purified components show that laminin binds through its short chains to native but not to denatured type IV collagen and through a domain in its long chain to the heparan sulfate proteoglycan (Terranova et al., 1980; Woodley et al., 1983; Rao et al., 1983). Alone each of these basement membrane components is soluble. When these macromolecules, however, are mixed together in vitro, they form a floccular precipitate containing laminin, type IV collagen, and heparan sulfate proteoglycan in a 1:1:0.1 molar ratio (Kleinman et al., 1983). However, this precipitate lacks the resiliency and consistency expected of basement membrane structures.

In this study, we have incubated extracts of the EHS tumor containing a mixture of proteins under physiological conditions and analyzed the components that interact and gel. These studies show that under physiological conditions certain components of the basement membrane including type IV collagen, laminin, heparan sulfate proteoglycan, nidogen, and entactin

interact in lamellar structures. These components of the matrix form supranuclear structures in the form of certain other

EXPERIMENTAL

Materials

Proteoglycan (1979; H. After the treatment with 1 M tris(hydrochloric acid), pH 7. 1976; Timpl extracted with Laminin was previously described from guanidine in interaction with to solubilize Low-density 6.0 M urea (1980; 1985) Co.

Unfractionated were prepared with high salt and 0.05 M trifluoroacetic acid with the same were combined Tris-HCl, pH 8.0, a Sorvall centrifuge insoluble material at 4°C in small described below. Extract was for collagen (0.1 mg/mL). E (Kleinman et al.) also present.

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Reconstitution. A centrifuge tube was added in solvent in 0.15 to the extract indicated. T with 0.15 M samples were was isolated in sample buffer acrylamide in experiment v

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act in rather constant proportions to form a gel with structures resembling in width those in basement membranes. Under the conditions employed here, each of the components appears to be required for the reconstitution of the matrix. It is proposed that the components of the gel are supramolecular complexes, which may be intermediates in the formation of the matrix. The reconstituted matrix promotes the growth and differentiation of melanoma and certain other cells.

EXPERIMENTAL PROCEDURES

Materials. Type IV collagen, laminin, and heparan sulfate proteoglycan were prepared from the EHS tumor (Timpl et al., 1979; Hassell et al., 1980, 1985; Kleinman et al., 1982). After the tumor tissue was washed in 3.4 M NaCl and 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, containing protease inhibitors (Orkin et al., 1976; Timpl et al., 1979), the basement membrane matrix was extracted with 0.5 M NaCl in 0.05 M Tris-HCl, pH 7.4. Laminin was isolated from the 0.5 M NaCl extract as previously described (Timpl et al., 1979). The residue of tumor tissue from lathyritic animals was extracted with 2.0 M guanidine in 0.05 M Tris-HCl, pH 7.4, followed by an extraction with the same buffer containing 0.005 M dithiothreitol to solubilize the type IV collagen (Kleinman et al., 1982). Low-density heparan sulfate proteoglycan was purified from 2.0 M urea extracts of the tumor by ion-exchange chromatography followed by cesium chloride density centrifugation and molecular sieve column chromatography (Hassell et al., 1980, 1985). Heparin was obtained from Sigma Chemical Co.

Unfractionated extracts of the basement membrane matrix were prepared by treating the tissue which had been washed with high salt with an equal volume (1 mL/g) of 2 M urea and 0.05 M Tris-HCl, pH 7.4, overnight at 4 °C and centrifuging at 10000g for 30 min. The residue was washed once with the same volume of buffer. Then the extract and wash were combined, dialyzed against 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.4 (TBS), and centrifuged at 15 000 rpm in a Sorvall centrifuge for 20 min to remove a small amount of insoluble material. The supernatant fraction was stored at -20 °C in small aliquots and used in the reconstitution assays described below. Using quantitative ELISA assays, this extract was found to contain laminin (3.5 mg/mL), type IV collagen (0.1 mg/mL), and heparan sulfate proteoglycan (0.1 mg/mL). Entactin, nidogen, and other minor components (Kleinman et al., 1982; J. R. Hassell, unpublished results) were also present. Entactin and nidogen were identified on the basis of their migration in sodium dodecyl sulfate (SDS) gels and cross-reactivity in Western blot analyses with antibodies obtained from A. Chung and R. Timpl, respectively. For column chromatography, the extract was dialyzed into 0.5 M NaCl and 0.05 M Tris-HCl, pH 7.4, and centrifuged at 40 000 rpm for 1 h to remove insoluble material.

Reconstitution Assays. Gelation was carried out in a centrifuge tube to which 0.05–0.1 mL of the 2 M urea extract was added in physiological buffer. Purified components dissolved in 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4, were added to the extract or were incubated together at the concentrations indicated. The final volume was made up to 0.5 or 1.0 mL with 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4, and the samples were incubated for 1 h at 35 °C. Insoluble material was isolated by centrifugation, and the pellets were dissolved in a sample buffer and electrophoresed in either 5% or 7.5% acrylamide under reducing conditions (Laemmli, 1970). Each experiment was repeated a minimum of 3 times. The total

amount of protein in the precipitate was determined by the Lowry procedure. The total amount of laminin was estimated by using the Lowry procedure but corrected for the relative amount of minor components present using scans of the gels. The amount of type IV collagen incorporated into the gel was quantitated by using ¹⁴C-labeled type IV collagen, and heparan sulfate proteoglycan incorporation was quantitated by using [³⁵S]sulfate-labeled material of known specific activities in parallel experiments. Estimates of the amount of nidogen and entactin in the gel were obtained by scanning negatives of photographs of the gels in a Helena densitometer and then related to the amount of laminin and total protein in the gel.

Rotary Shadowing. The 2.0 M urea extract equilibrated in 0.5 M NaCl and 0.05 M Tris-HCl, pH 7.4, was placed on a Sepharose 4B column. An aliquot (30 µL) of the peak fraction (0.1 mg/mL) eluting from the column was diluted with 300 µL of 0.155 M ammonium acetate, pH 7.4, and 600 µL of glycerol. For rotary shadowing, the mixture was sprayed onto mica, shadowed with platinum-palladium, carbon coated, and examined in a JEOL 100C electron microscope.

Ultrastructure of Reconstituted Components. The gel was prepared essentially as described above. Briefly, 0.2 mL of the extract was incubated alone or in the presence of type IV collagen and heparan sulfate proteoglycan overnight at 35 °C. The gel was isolated by centrifugation at 10 000 rpm for 10 min and then fixed in 2.5% glutaraldehyde, treated with 1% OsO₄, block-stained with 2% uranyl acetate, and dehydrated. The gel was then processed through Epon for electron microscopy. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100C electron microscope. Thin sections of rat kidney tubule basement membranes were obtained as previously described (Laurie et al., 1984b).

Cell Culture. B16C3 cells were cultured either directly on tissue culture plastic or on a 1-mm-thick basement membrane gel in a mixture of F12 medium and Dulbecco's modified Eagle's medium (lacking phenol red for visualization of the pigmentation of the cells) containing glutamine, antibiotics, 20 mM tyrosine, and 5% fetal calf serum. After 1 week, the cells were photographed.

RESULTS

The assembly of basement membrane components was analyzed by using purified basement membrane components as well as unfractionated extracts of basement membrane. Purified type IV collagen, laminin, and heparan sulfate proteoglycan formed a flocculent precipitate when incubated under physiological conditions for 1 h at 35 °C (Kleinman et al., 1983). In contrast, a gel formed when urea extracts of basement membrane are dialyzed against physiological saline and then warmed to 35 °C for 1 h. The components of the gel were isolated by centrifugation and examined by SDS gel electrophoresis. The amount of laminin, entactin, and nidogen present in the gel was found to increase as increasing amounts of type IV collagen were added until some 50% of the material in the extract was incorporated into the gel (Figure 1A, C). Heparan sulfate proteoglycan also caused increasing amounts of basement membrane components to precipitate (Figure 1B). Separation by gel electrophoresis and determination of the relative amounts of major components in the gel by scanning the negatives of the gels indicated that constant ratios of laminin, entactin, and nidogen are obtained in the presence of added type IV collagen (Figure 1C) or of heparan sulfate proteoglycan (data not shown). When both type IV collagen (150 µg) and heparan sulfate proteoglycan (10 µg) were added to the extract, up to 80% of the protein present was incorporated into the gel (data not shown). Since the smaller chains

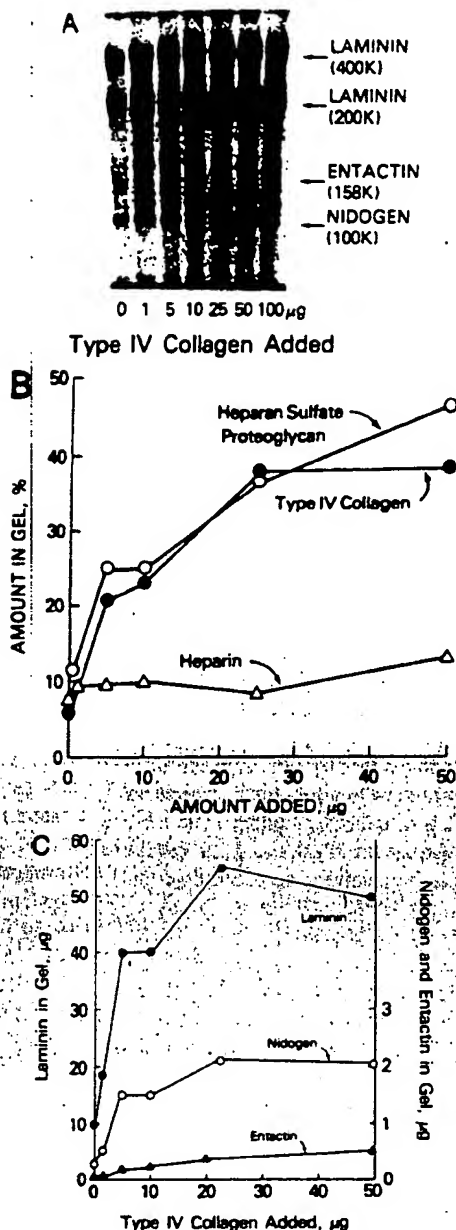


FIGURE 1: Effect of type IV collagen, heparan sulfate proteoglycan, and heparin on the gelation of basement membrane components from the basement membrane extract. Increasing amounts of each component were added to 100 μ L of the extract and incubated for 1 h at 35 $^{\circ}$ C in 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4. The samples were then centrifuged, and the pellet was dissolved in sample buffer. Equal aliquots of the samples were electrophoresed in 5% acrylamide, fixed, and stained with Coomassie blue. (A) Effect of type IV collagen on the amount of protein in the gel. (B) Quantitative effects of type IV collagen, heparan sulfate proteoglycan, and heparin on the amount of protein present in the gel. The Lowry assay was used to determine the amount of protein. (C) Effect of type IV collagen on the amount of laminin, nidogen, and entactin in the gel. Densitometric scans of the SDS gels were used to determine the relative amounts of these components in the gel. The amounts of nidogen and entactin were estimated on the basis of their relative density and the total protein in the gel.

of laminin coelectrophoresed with the chains of type IV collagen and prevented its visualization in the SDS gel, we used 3 H-labeled type IV collagen of known specific activity in separate experiments to measure the amount of type IV collagen. Likewise, since heparan sulfate proteoglycan cannot be visualized in the gels, 35 S-labeled heparan sulfate proteoglycan was used. These studies showed that in a typical ex-

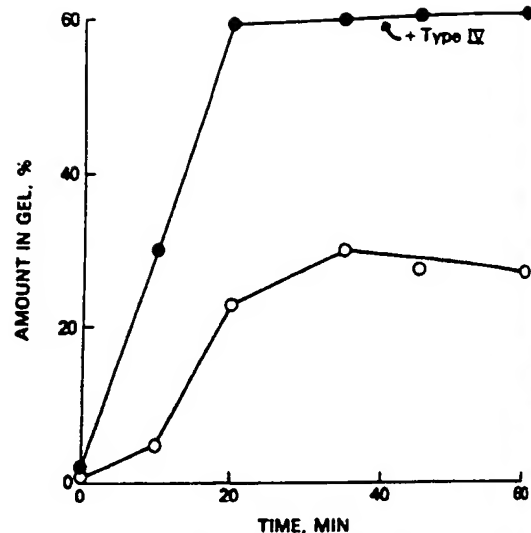


FIGURE 2: Effect of time and added type IV collagen on the gelation of the basement membrane extract. The conditions are similar to those described in the legend for Figure 1. This figure compares gelation in the presence (●) and absence (○) of type IV collagen (50 μ g).

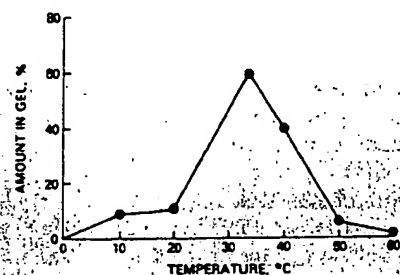


FIGURE 3: Effect of temperature on the gelation of extracts of basement membrane. The experiment was carried out in the presence of type IV collagen (50 μ g) as described in the legend for Figure 1. Gelation was stopped by centrifugation at the times indicated.

periment laminin accounted for almost 60% (264 ± 56 μ g) of the material in the gel, type IV collagen for 30% (125 ± 7 μ g), heparan sulfate proteoglycan for less than 3% (8.3 ± 0.7 μ g), nidogen for 5%, and entactin for less than 1%. These values are comparable to the proportions of laminin (15 mg/g wet weight), type IV collagen (8 mg/g), and heparan sulfate proteoglycan (1 mg/g) present in the EHS tumor (Kleinman et al., 1982). In contrast, supplementation of the extract with either type I collagen, fibronectin (data not shown), or heparin (Figure 1B) did not cause any increased precipitation, indicating that specific interactions are involved. Removal of the protein core of the proteoglycan by incubation overnight with 0.5 N NaOH destroyed its ability to induce polymerization, suggesting that the protein portion of the proteoglycan is involved in binding to other components (data not shown).

Under physiological conditions, the gelation process is complete within 20 min (Figure 2). The formation of the gel is strongly dependent on temperature with maximum polymerization at 35 $^{\circ}$ C (Figure 3). The lack of interaction at 0 $^{\circ}$ C suggests that thermal denaturation inactivates a critical constituent.

The stability of the gel to dissolution was examined by various solvents. The gel was not dissolved by cold aqueous salt (0.15 or 0.5 M NaCl) but was partially dissolved by boiling solutions (43% solubilized) and almost completely dissolved in 2.0 M guanidine (97%) or in 2.0 M urea solutions. This suggests that the components are linked by relatively strong but noncovalent bonds. When the guanidine-dissolved gel was

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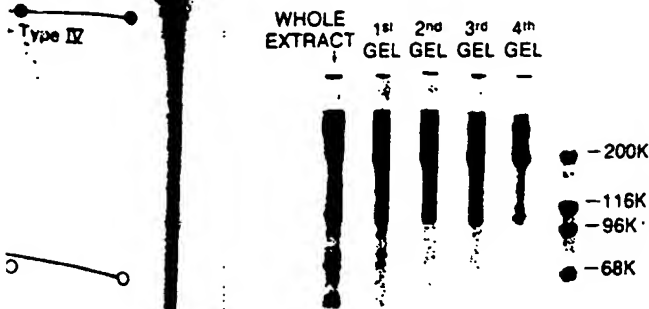
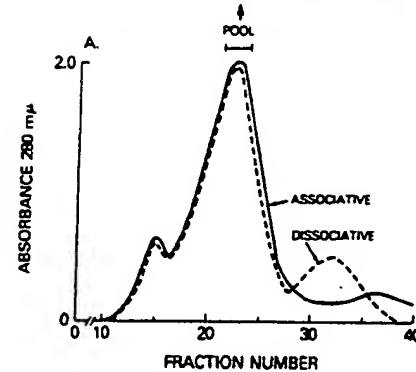
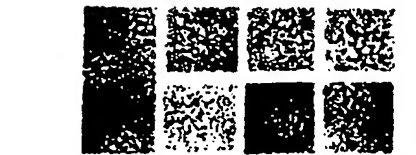


FIGURE 4: Ability of the basement membrane extract to regel following dissolution of the gel. The first lane, designated "whole extract", demonstrates the components in the starting material. The "1st gel" designates the components in the gel formed in the presence of type IV collagen. The material present in the gel formed in the absence of type IV collagen (not shown) was solubilized for 20 min in 2.0 M guanidine, dialyzed against 0.05 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, and allowed to regel in the absence (not shown) and presence of type IV collagen (designated "2nd gel"). The cycle was repeated additional times ("3rd gel" and "4th gel"). Shown are equal aliquots of the gels electrophoresed in a 5% acrylamide gel.

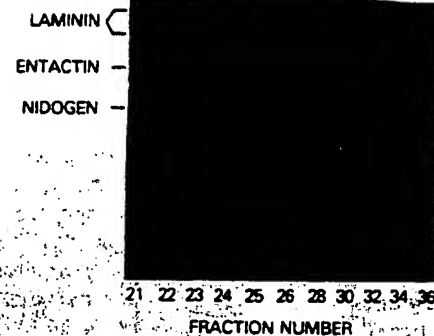
analyzed against physiological buffers and warmed in the presence of type IV collagen, gellike structures were reconstituted. This process could be repeated several times with similar proportions of laminin, nidogen, and entactin depositing at each step as determined by SDS-polyacrylamide gels (Figure 4). Densitometric scans of the gels indicated that laminin and type IV collagen were 94% of the total material and nidogen and entactin represented 5% and 1%, respectively. In the presence of added type IV collagen, re-formation of the gel occurred more rapidly, and greater amounts of the components were deposited (data not shown).

We next determined whether soluble complexes of basement membrane components existed. When the urea extract was dialyzed free of urea and passed over a Sepharose 4B column in 0.5 M NaCl (associative conditions), laminin, nidogen, and entactin eluted in a major included peak (Figure 5A,B). When the material in the major included peak was pooled and run over the same molecular sieve column in 4 M guanidine (dissociative conditions), these components separated in the manner expected from their molecular weights (Figure 5A,C). These results indicate that there are strong but noncovalent bonds joining laminin, nidogen, and entactin in the complex. Rotary shadowing electron microscopy of the major included peak material confirmed the presence of soluble complexes (Figure 5A). The complexes involved the proteoglycan which appears as a large globule due to collapse of the heparan sulfate side chains in this kind of preparation (G. W. Laurie et al., unpublished results) surrounded by several laminin molecules. The nidogen and entactin molecules could not be distinguished in the electron micrographs but are known to be in the complexes from the electrophoresis study (Figure 5B).

The ultrastructure of the reconstituted basement membrane both with and without type IV collagen and heparan sulfate proteoglycan was examined. In the absence of added type IV collagen and heparan sulfate proteoglycan, the gel consisted of numerous widely separated thin, filamentous aggregates (Figure 6a). The addition of type IV collagen (not shown) and heparan sulfate proteoglycan plus type IV collagen (Figure 6b) resulted in the formation of thin sheets which were interconnected (Figure 6b) or were confluent. The individual segments of the network had an average width similar to that of the lamina densa of kidney tubule basement membrane (Figure 6c). However, unlike native basement membranes in which lamina densalike layers are arranged in parallel, such as, for example, the PYS tumor basement membranes



B. ASSOCIATIVE



C. DISSOCIATIVE

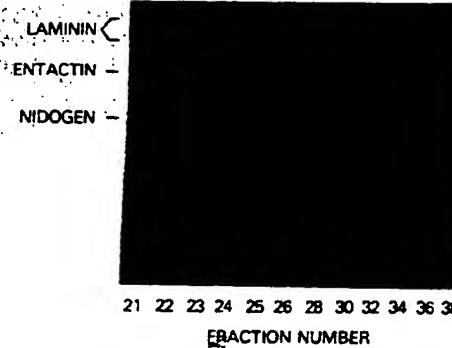


FIGURE 5: Sepharose 4B column chromatography of the 2.0 M urea extract. Two milliliters of the whole extract equilibrated in either 2 M urea, 0.15 M NaCl, and 0.05 M Tris, pH 7.4 (dissociative), or 0.5 M NaCl and 0.05 M Tris, pH 7.4 (associative), was placed on a Sepharose 4B column (2 x 60 cm) equilibrated in the corresponding buffer. (A) Aliquots of the designated fractions from the extract chromatographed in associative (B) or dissociative (C) conditions were analyzed by SDS-polyacrylamide gels. In addition, an aliquot of the material eluting from the column run under associative conditions was examined in the electron microscope by rotary shadowing (A). The electron micrographs show that the most common complex in the peak fractions involves a central heparan sulfate proteoglycan and numerous peripheral laminin molecules. Entactin and nidogen are not readily visualized in these complexes.

(Martinez-Hernandez et al., 1982) or Reichert's membrane (Inoue et al., 1983), the lamina densalike structures were interconnected and did not form parallel multilamellar structures. At very high power in the electron microscope, each segment could be resolved into 5-nm cords as previously described in other basement membranes (Inoue et al., 1983; Laurie et al., 1984).



FIGURE 6: Electron micrographs of reconstituted gels and an authentic basement membrane. (a) Gel formed in the absence of added type IV collagen or heparan sulfate proteoglycan. The gel consists of dispersed segments with occasional interconnections. (b) Gel formed in the presence of added type IV collagen and heparan sulfate proteoglycan. The edge of the gel is at the top. The gel consists of an interconnected network; the network is made up of structures which are similar in width to the lamina densa part of native basement membranes. These lamina densalike structures vary somewhat in thickness. (c) Kidney tubule basement membrane from a 100-g rat. The basement membrane consists of the lamina lucida and lamina densa. Extensions from the lamina densa attach it to the cell membrane (arrowheads). Bar = 200 μ m; 42750X.

The reconstituted basement membrane was used to coat the surfaces of bacteriological petri dishes and tested as a substrate for the growth and differentiation of various cells. Melanoma cells (B16C3) showed considerable differences in morphology when grown on the basement membrane gel as compared to

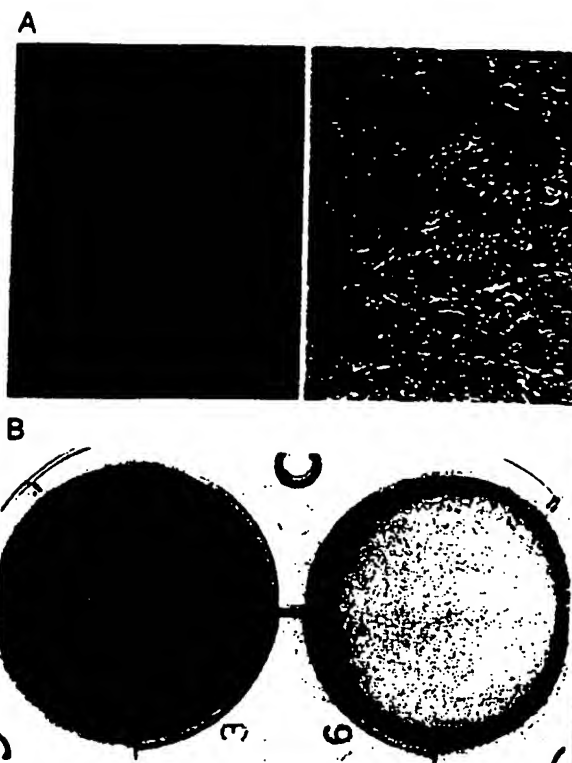


FIGURE 7: Effect of the basement membrane gel on the morphology and differentiation of B16C3 melanoma cells in culture. Sterile 1 M urea extract of the EHS tumors in 0.15 M NaCl and 0.05 M Tris-HCl, pH 7.4, was allowed to gel on the surface of a petri dish for 30 min at 37 °C. Then equal numbers of cells were plated on the gel (left) or onto control tissue culture plastic dishes (right). After 1 week in culture in DMEM containing 20 mM tyrosine, gentamicin, glutamine, and 5% fetal calf serum, the cells were photographed. (A) Morphology and assessment of melanogenesis by the cells. (B) Direct view of the dishes. The gel at the edge has been deflected to show that the cells are attached to it.

tissue culture surfaces (Figure 7). Further, there was a much earlier and more extensive pigmentation of the cells on this substrate. Preliminary studies of other cells showed that endothelial cells formed tubelike structures on the gel (G. Grotendorst, unpublished results) and that hepatocytes survived longer on basement membrane gel substrates than on tissue culture plates or on type I collagen (L. Reid, unpublished results). In vivo, the basement membrane gel was found to promote peripheral nerve regeneration (Madison et al., 1985). Such studies indicate that the reconstituted basement membrane is a biologically active substrate which induces diverse cellular responses. Since it can support cell adhesion, growth, and differentiation beyond that reported for the individual components, it is likely that the reconstituted basement membrane gel contains these molecules in an active and synthetic conformation.

DISCUSSION

It is now well appreciated that, while collagens form the major structural element of extracellular matrices, glycoproteins and proteoglycans are also important constituents regulating many other activities including cell-matrix interactions (Hay, 1982; Timpl & Martin, 1982). Different matrices contain different sets of these matrix proteins which together contribute to the unique physical and biological characteristics of tissues.

In studies on the constituents of basement membranes, we have observed that they form supramolecular complexes (Kleinman et al., 1983). The individual components, type IV

collagen, laminin, and heparan sulfate proteoglycan, interact with each other. These observations indicate that the interaction of these components cannot be separated from the network. The network is linked through the laminin component (Bächinger, 1982). The laminin component is a large open network (Yurchenco, 1982). It is presumed that the laminin component is concurrent with the collagen component. In a basement membrane, the type IV collagen component, does not form the gel, the laminin component (unpublished).

In this study, we have shown that the additional components of the basement membrane, i.e., laminin and heparan sulfate proteoglycan, are essential for the formation of a basement membrane. The laminin component is a maximal component of the basement membrane structure. The laminin component is a maximal component of the basement membrane structure.

Experiments have shown that these interactions are important for the formation of a basement membrane. The laminin component is a maximal component of the basement membrane structure. The laminin component is a maximal component of the basement membrane structure.

Molecular models of the basement membrane have been proposed. These models are based on the observation that the basement membrane is a complex of various components. The laminin component is a maximal component of the basement membrane structure. The laminin component is a maximal component of the basement membrane structure.

laminin, and heparan sulfate proteoglycan, are soluble they precipitate when incubated in combination. These observations have led us to suggest that cooperative interactions are involved in their deposition into the basement membrane. This is not to say that the individual components do not self-assemble. Type IV collagen molecules obtained from the media of cultured cells form tetrameric aggregates through the amino-terminal portion of the molecule (Bächinger et al., 1982; Fessler & Fessler, 1982). Dimeric components of type IV collagen such as used here will form a large open network, while laminin will also self-aggregate (Yurchenco & Furthmayr, 1984; Yurchenco et al., 1984). We assume, however, that these components are synthesized independently and that cooperative interactions may predominate. In addition, such interactions are not likely to be important in the initial interactions since prior incubation of the type IV collagen, under conditions where type IV self-assembly does not affect the amount of material incorporated into the gel, the rate of gel formation, or the appearance of the gel (published observations).

In this study, we have attempted to determine whether additional components are involved in the formation of the basement membrane and whether these are specific and stoichiometric interactions. In these studies, the basement membrane of EHS tissues was extracted with a chaotropic agent (2 M urea) to disrupt noncovalent interactions and to solubilize various glycoproteins and proteoglycans into solution. Only small amounts of type IV collagen are present in the extract since it is cross-linked by disulfide bonds and by lysine-derived cross-links. Some of the material in the extract formed a gel when the solution was dialyzed against physiological saline and brought to 37°C. However, when type IV collagen was added to the extract, there was an increased deposition of laminin, nidogen, and entactin. The addition of type IV collagen plus heparan sulfate proteoglycan leads to maximal incorporation of these compounds into a gellike structure containing interconnected thin sheets.

Experiments described here and previously suggest that these interactions are specific (Terranova et al., 1980; Woodley et al., 1983; Kleinman et al., 1983). For example, type I collagen cannot substitute for type IV collagen, nor fibronectin for laminin nor heparin for heparan sulfate proteoglycan. Such specificity is expected and parallels the known affinities of these components for binding to one another. In addition, there appears to be a stoichiometric relation in the interaction of these materials. When redissolved, the components of the gel constitute in the same proportions. Such behavior is expected if these interactions occur through a limited number of specific binding sites as suggested in studies on their interactions (Rao et al., 1983; Mai & Chung, 1984; Laurie et al., 1984a; Dziadek & Timpl, 1985). However, it is possible that nidogen and entactin and perhaps other minor components of the basement membrane may be involved in organizing the materials that precipitate into gellike and sheetlike structures.

Molecular sieve studies also supported the existence of supramolecular complexes which were dissociable in guanidine, and these complexes were visualized directly after rotary shadowing by electron microscopy. The major features of these complexes appear to be a central core of proteoglycan surrounded by several laminin molecules. Laminin alone does not form such complexes but will when incubated with heparan sulfate proteoglycan (G. W. Laurie et al., unpublished results). Nidogen and entactin could not be distinguished in these preparations perhaps because they are much smaller than the other constituents. Taken together, these studies indicate that

laminin, nidogen, and entactin form defined complexes and that type IV collagen and heparan sulfate proteoglycan cause them to be incorporated into an insoluble gellike structure.

Supramolecular complexes of matrix components have been observed in other systems and have been shown to consist of procollagen, proteoglycan, and fibronectin (Schubert & La-Corbiere, 1980; Hedman et al., 1982; Schubert et al., 1983). Again, these materials are produced by the same cell and may even be secreted together. Thus, it is possible that supramolecular complexes of matrix components arise in a variety of sites and have a role in the formation of as well as in determining the composition of newly deposited extracellular matrices.

Since matrix substrates affect the behavior of cultured cells, we tested the growth and differentiation of cells on the reconstituted basement membrane. To date, we and others to whom we have supplied the gel have found rather strong effects on the growth and differentiation of cultured cells. As shown here, melanocytes aggregate into clusters and show an accelerated melanogenesis. Hadley et al. (1985) have found that dissociated embryonic Sertoli cells will reorganize in the gel into tubular structures which resemble in organization the original tissue. The gel also encourages the in vivo growth of neurons (Madison et al., 1985) and the in vitro growth and differentiation of Schwann cells (D. Carey and M. Todd, unpublished results) and liver cells (L. Reid, unpublished results). In general, epithelial cells assume a much greater polarity on this substrate than on plastic, collagen, laminin, or fibronectin substrates.

The ability of complex substrates to support cell growth and differentiation is well documented [see, for example, Dodson & Hay (1974), Rojkind et al. (1980), Gospodarowicz & Lui (1981), Lillie & MacCallum (1982), Mai & Chung (1984), and Wicha et al. (1982)]. Cells appear to have specific receptors not only for glycoproteins such as laminin and fibronectin but also for collagen and proteoglycans (Goldberg, 1979; Rubin et al., 1978; Kurkinen et al., 1984; Bernfield & Banerjee, 1982; Lattera et al., 1980). Presumably cells function best on their natural matrix with each cellular receptor bound to the appropriate matrix component.

ADDED IN PROOF

Since this paper was prepared, it has been found that nidogen has higher molecular weight forms similar in size to entactin. Thus, entactin and nidogen may be related proteins.

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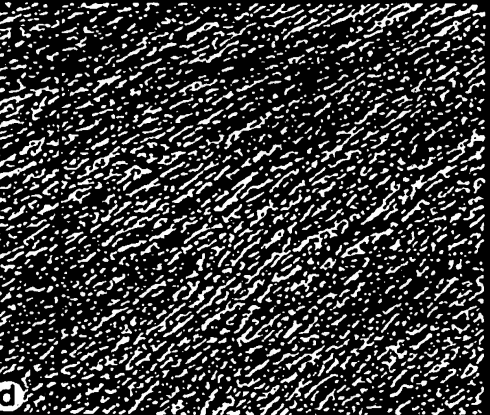
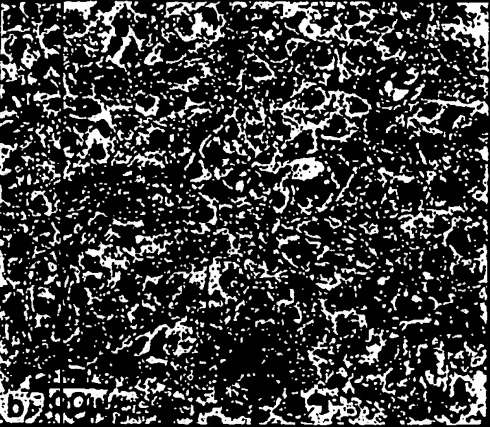
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CULTURE OF ANIMAL CELLS

A Manual of
Basic Technique

R. Ian Freshney

AV

Alan R. Liss, Inc., New York

Maintenance of the Culture—Cell Lines

Chapter 12

The first subculture represents an important transition for the culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrate. Hence cell proliferation has become an important feature. While the primary culture may have variable growth fraction (see Chapter 19) depending on the type of cells present in culture, after the first subculture, the growth fraction is usually high (80% or more).

From a very heterogeneous primary culture, containing many of the cell types present in the original tissue, a more homogeneous cell line emerges. In addition to its biological significance, this has also considerable practical importance as the culture can now be propagated, characterized, and stored, and the potential increase in cell number and uniformity of the cells opens up a much wider range of experimental possibilities.

Once a primary culture is subcultured (or "passed," or "transferred"), it becomes known as a "cell line." This term implies the presence of several cell lineages either of similar or distinct phenotypes. If one cell lineage is selected, by cloning (see Chapter 13), by physical cell separation (see Chapter 14), or by any other selection technique, with certain specific properties which have been identified in the bulk of the cells in the culture, this becomes known as "cell strain."

NOMENCLATURE

The first subculture gives rise to a "secondary" culture, the secondary to a "tertiary" and so on, although in practice this nomenclature is seldom used beyond tertiary. Since the importance of culture lifetime was highlighted by Hayflick and others with diploid fibroblasts [Hayflick and Moorhead, 1961], where each subculture divided the culture in half ("split ratio" = 1:2), passage number has often been confused

with "generation number." Cell lines with limited culture life-spans ("finite" cell lines) behave in a fairly reproducible fashion (see Chapter 2). They will grow through a limited number of cell generations, usually between 20 and 80 population doublings, before extinction. The actual number depends on strain differences and culture conditions but is consistent for one cell line grown under the same conditions. It is, therefore, important that reference to a cell line should express the approximate generation number or number of doublings since explantation, "approximate" because the number of generations which have elapsed in the primary culture is difficult to assess.

The cell line should also be given a code or designation (e.g., NHB, normal human brain), a cell strain or cell line number (if several cell lines were derived from the same source) NHB1, NHB2, etc., and if cloned, a clone number, NHB2-1, NHB2-2, etc. Together with the number of population doublings, this becomes, for example, NHB2/2 and will increase by one for a split ratio of 1:2 (NHB2/2, NHB2/3, etc.), by a two for a split ratio of 1:4 (NHB2/2, NHB2/4, etc.), and so on. For publication, each cell line should be prefixed with a code designating the laboratory in which it was derived, e.g., W1, Wistar Institute [Fedoroff, 1975].

ROUTINE MAINTENANCE

Once a culture is initiated, whether it be a primary culture or a subculture of a cell line, it will need a periodic medium change ("feeding") followed eventually by subculture if the cells are proliferating. In nonproliferating cultures, the medium will still need to be changed periodically as the cells will still metabolize, and some constituents of the medium will become exhausted or will degrade spontaneously. Intervals between medium changes and between subcultures vary from one cell line to another depending on the rate of

growth and metabolism; rapidly growing cell lines such as HeLa are usually subcultured once per week and the medium changed 4 d later. More slowly growing cell lines may only require to be subcultured every 2, 3, or even 4 wk, and the medium changed weekly between subcultures. (For a more detailed discussion of the growth cycle, see below.)

Replacement of Medium

Four factors indicate the need for the replacement of culture medium (see also Chapter 9).

A drop in pH. The rate of fall and absolute level should be considered. Most cells will stop growing as the pH falls from pH 7.0 to pH 6.5 and will start to lose viability between pH 6.5 and pH 6.0, so if the medium goes from red through orange to yellow, the medium should be changed. Try to estimate the rate of fall: a culture which falls at 0.1 pH units in 1 d will not come to harm if left a day or two longer before feeding, but a culture that falls 0.4 pH units in 1 d will need to

be fed 24–48 hr later and cannot be left over a weekend.

Cell concentration. Cultures at a high cell concentration will use up the medium faster than at a low concentration. This is usually evident in the pH change but not always.

Cell type. Normal cells (e.g., diploid fibroblasts) will usually stop dividing at high cell concentrations (density limitation of growth; see Chapter 16) due to growth factor depletion and other factors. The cells block in the G1 phase of the cell cycle and deteriorate very little even if left for 2–3 wk. Transformed cells, continuous cell lines, and some embryonic cells, however, will deteriorate rapidly at high cell densities unless the medium is changed daily or they are subcultured.

Cell morphology. When checking a culture for routine maintenance, be alert to signs of morphological deterioration: granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with detachment from the substrate (Fig. 12.1). This may



Fig. 12.1. Signs of deterioration of the culture. Cytoplasm of cells becomes granular, particularly around the nucleus, and vacuolation occurs. Cells may become more refractile at the edge if cell spreading is impaired.

imply that the culture requires a medium change, or may indicate a more serious problem, e.g., inadequate or toxic medium or serum, microbiological contamination, or senescence of the cell line. During routine maintenance, the medium change or subculture frequency should prevent such deterioration.

Volume, Depth, and Surface Area

The usual ratio of medium volume to surface area is 0.2–0.5 ml cm². The upper limit is set by gaseous diffusion through the liquid layer and the optimum will depend on the oxygen requirement of the cells. Cells with a high O₂ requirement will be better in shallow medium (2 mm) and those with a low requirement may do better in deep medium (5 mm). If the depth is greater than 5 mm, then gaseous diffusion may become limiting. With monolayer cultures this can be overcome by rolling the bottle or perfusing the culture with medium and arranging for gas exchange in an intermediate reservoir (see Chapter 21). When the depth of suspension culture is increased, it should be stirred with a bar magnet (see Chapter 21). To prevent frothing, the depth of stirrer cultures must be a minimum of 5 cm. For intermediate depths of medium between 5 mm and 5 cm, use a roller bottle (see Table 8.1).

“Holding Medium”

A holding medium may be used where stimulation of mitosis, which usually accompanies a medium change, even at high cell densities, is undesirable. Holding media are usually regular media with the serum concentration reduced to 1 or 2%. This will not stimulate mitosis in most untransformed cells. Transformed cell lines are unsuitable for this procedure as they may either continue to divide successfully or the culture may deteriorate as transformed cells do not block in a regulated fashion in G₁.

Holding medium is also used to maintain cell lines with a finite life-span without using up the limited number of cell generations available to them (see Chapter 2). Reduction of serum and cessation of cell proliferation also promotes expression of the differentiated phenotype in some cells [Schousboe et al., 1979; Maltese and Volpe, 1979].

Changing the Medium or “Feeding” a Culture

Outline

Examine the culture on an inverted microscope. Then, if indicated, remove the old medium and add

fresh medium. Return the culture to the incubator.

Materials

Pipettes
medium
(both sterile)

Protocol

1. Examine culture carefully for signs of contamination or deterioration (see Fig. 17.1 and 12.1)

2. Check the criteria described above—pH, cell density, or concentration, and, based on your knowledge of the behavior of the culture, decide whether or not to replace the medium. If feeding is required, proceed as follows

3. Take to sterile work area, remove and discard medium (see Chapter 5)

4. Add same volume of fresh medium, prewarmed to 36.5°C if it is important that there is no check in cell growth

5. Return culture to incubator

Note:

Where a culture is at a low density and growing slowly, it may be preferable to “half-feed.” In this case, remove only half the medium at step 3 and replace it in step 4 with the same volume as was removed.

Subculture

When all the available substrate is occupied, or when the cell concentration exceeds the capacity of the medium, either the frequency of medium changing must increase or the culture must be divided. The usual practice in subculturing an adherent cell line involves removal of the medium and dissociation of the cells in the monolayer with trypsin, although some loosely adherent cells (e.g., HeLa or Chang liver) may be subcultured by shaking the bottle and collecting the cells in the medium, and diluting as appropriate in fresh medium in new bottles. Exceptionally, some cell monolayers cannot be dissociated in trypsin and require the action of alternative proteases such as pronase, dispase, or collagenase (Table 12.1) [Foley and Aftonomos, 1973].

TABLE 12.1. Cell Dissociation for Transfer or Counting—Procedures of Gradually Increasing Severity

1. Shake-off	Mitotic or other loosely adherent cells
2. Trypsin* in PBS (0.01–0.5% as required, usually 0.25%, 5–15 min)	Most continuous cell lines
3. Prewash with PBS or CMF, then 0.25% trypsin* in PBS or saline-citrate	Some strongly adherent continuous cell lines and many cell lines at early passage stages
4. Prewash with 1 mM EDTA in PBS or CMF then 0.25% trypsin* in citrate	Some strongly adherent early passage cell lines
5. Prewash with 1 mM EDTA, then EDTA 2nd rinse, and leave on, 1 ml/5 cm	Epithelial cells, although some may be sensitive to EDTA
6. EDTA prewash, then 0.25% trypsin* with 1 mM EDTA	Strongly adherent cells, particularly epithelial and some tumor cells (note: EDTA can be toxic to some cells)
7. 1 mM EDTA prewash, then 0.25% trypsin* and collagenase* 200 units/ml PBS or saline-citrate or EDTA/PBS	Thick cultures, multilayers, particularly collagen-producing dense cultures
8. Scraping	All cultures, but may cause mechanical damage and usually will not give a single cell suspension
9. Add dispase (0.1–1.0 mg/ml) or pronase (0.1–1.0 mg/ml) to medium and incubate till cells detach	Will dislodge most cells, but requires centrifugation step to remove enzyme not inactivated by serum. May be harmful to some cells.

*Digestive enzymes are available (Difco, Worthington, Boehringer Mannheim, Sigma) in varying degrees of purity. Crude preparations, e.g., Difco trypsin 1:250 or Worthington CLS grade collagenase, contain other proteases which may be helpful in dissociating some cells but may be toxic to others. Start with a crude preparation and progress to purer grades if necessary. Purer grades are often used at a lower concentration (mg/ml) as their specific activities (enzyme units/g) are higher. Purified trypsin at 4° C has been recommended for cells grown in low serum concentrations or in the absence of serum [McKeehan, 1977].

The attachment of cells to each other and to the culture substrate is mediated by cell surface glycoproteins and Ca^{2+} and Mg^{2+} ions. Other proteins, derived from the cells and from the serum, become associated with the cell surface and the surface of the substrate and facilitate cell adhesion.

Outline

Remove medium, expose cells briefly to trypsin, incubate and disperse cells in medium.

Materials

Pipettes (sterile)
medium (sterile)
PBSA (sterile)
0.25% trypsin in PBSA, saline citrate, or EDTA (sterile) (see Table 12.1)
culture flasks
hemocytometer or cell counter

Protocol

1. Withdraw medium and discard
 2. Add PBSA prewash (5 ml/25 cm²) to the side of the flask opposite the cells, so as to avoid dislodging cells, rinse the cells, and discard rinse. This step is designed to remove traces of serum which would inhibit the action of the trypsin
 3. Add trypsin (3 ml/25 cm²) to the side of the flask opposite the cells. Turn the flask over to cover the monolayer completely. Leave 15–30 sec and withdraw the trypsin, making sure beforehand that the monolayer has not detached. Using trypsin at 4° C helps to prevent this
 4. Incubate until cells round up; when the bottle is tilted, the monolayer should slide down the surface (this usually occurs after 5–15 min). Do not leave longer than necessary, but do not force the cells to detach before they are ready to do so, or clumping may result
- Note.
- If difficulty is encountered in getting cells to detach, and, subsequently, in preparing a single cell suspension, alternative procedures, as described in Table 12.1, may be employed. In each case the main dissociating agent, be it trypsin or EDTA, is present only briefly and the incubation is performed in the residue after most of the dissociating agent has been removed.
5. Add medium (0.1–0.2 ml/cm²) and disperse cells by repeated pipetting over the surface bearing the monolayer. Finally, pipette the cell suspension up and down a few times, with the tip of the pipette resting on the bottom corner of bottle. The degree of pipetting required will vary from one cell line to

another; some disperse easily, others require vigorous pipetting. Almost all will incur mechanical damage from shearing forces if pipetted too vigorously; primary suspensions and early passage cultures are particularly prone to damage due partly to their greater fragility and partly to their larger size. Pipette up and down sufficiently to disperse the cells into a single cell suspension. If this is difficult, apply a more aggressive dissociating agent (see Table 12.1) [Toshiharu et al., 1975]

A single cell suspension is desirable at subculture to ensure an accurate cell count and uniform growth on reseeding. It is essential where quantitative estimates of cell proliferation or of plating efficiency are being made and where cells are to be isolated as clones

6.

Count cells by hemocytometer or electronic particle counter (see Chapter 19)

7.

Dilute to the appropriate seeding concentration (a) by adding the appropriate volume of cells to a premeasured volume of medium in a culture flask, or (b) by diluting the cells to the total volume required and distributing that among several flasks. Procedure (a) is useful for routine subculture when only a few flasks are used and precise cell counts and reproducibility are not critical, but (b) is preferable when setting up several experimental replicate samples, as the total number of manipulations is reduced and the concentration of cells in each flask will be identical

8.

Cap the flask(s) and return to the incubator. Check after about 1 hr for pH change. If the pH rises, return to aseptic area and gas culture lightly with 5% CO₂. Since each culture will behave predictably in the same medium, you will know eventually which cells to gas when they are passaged, without having to incubate them first.

Expansion of air inside plastic flasks causes the flasks to swell and prevents them from lying flat. Release the pressure by slackening the cap briefly. This may be prevented by compressing the top and bottom of large flasks before sealing them. Incubation then restores the correct shape. Care must be taken not to crack the flasks

For finite cell lines, it is convenient to reduce the cell concentration at subculture by two-, four-, eight-, or 16-fold, making the calculation of the number of population doublings easier ($2 \equiv 1$, $4 \equiv 2$, $8 \equiv 3$,

$16 \equiv 4$). e.g., a culture divided eightfold will require three doublings to achieve the same cell density. With continuous cell lines, where generation number is not usually recorded, the cell concentration is more conveniently reduced to a round figure, e.g., 5×10^4 cells/ml. In both cases, the cell count should be recorded so that growth rate can be estimated at each subculture and consistency monitored (see below under Growth cycle).

Propagation in Suspension

The preceding instructions refer to subculture of monolayers, as most primary cultures or continuous lines grow in this way. Cells which grow continuously in suspension, either because they are nonadhesive (e.g., many mouse leukemias and ascites tumors) or because they have been kept in suspension mechanically, or selected (see also Chapter 21), may be subcultured like micro-organisms. Trypsin treatment is not required and the whole process is quicker and less traumatic for the cells. Medium replacement is not usually carried out with suspension cultures as this would require centrifugation of the cells. Routine maintenance is therefore reduced to one of two alternative procedures, i.e., subculture by dilution, or increase of the volume without subculture.

Outline

Count cells, withdraw cell suspension, and add fresh medium to restore cell concentration to starting level.

Materials

Culture flasks (sterile)
medium (sterile)
pipettes (sterile)
bar magnet (sterile)
magnetic stirrer
hemocytometer or cell counter

Protocol

1.
Mix cell suspension and disperse any clumps by pipetting
 2.
Remove sample and count
 3.
Add medium to fresh flask
- Note.
Any culture flask with a reasonably flat surface

may be used for cells which grow spontaneously in suspension. Where stirring is required, e.g., for larger cultures, or cells which would normally attach, use standard round reagent bottles, or aspirators, siliconized, if necessary (see Appendix), and insert a magnetic stirrer bar, Teflon coated, and with a ridge around the middle (Fig. 12.2) or suspended from the top of the bottle. Select the appropriate size of bottle to give between 5 and 8 cm depth with the volume of medium that you require

4.

Add sufficient cells to give a final concentration of 10^5 cells/ml for slow-growing cells (24–48-hr doubling time) or 2×10^4 /ml for rapidly growing cells (12–18-hr doubling time)

5.

Cap and return culture to incubator

6.

Culture flasks should be laid flat as for monolayer culture. Stirrer bottles should be placed on a magnetic stirrer and stirred at 60–100 rpm. Take care that the stirrer motor does not overheat the culture. Insert a polystyrene foam mat under the bottle, if necessary. Induction-drive stirrers generate less heat and have no moving parts

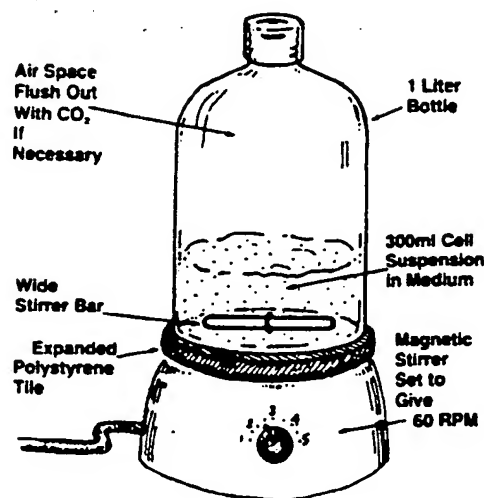


Fig. 12.2. Simple stirrer culture for cells growing in suspension. An expanded polystyrene mat (dark shaded area below bottle) should be interposed between the bottle and the magnetic stirrer to avoid heat transfer from the stirrer motor.

Suspension cultures have a number of advantages (see Table 12.2). The production and harvesting of large quantities of cells may be achieved without increasing the surface area of the substrate (see Chapter 21). Furthermore, if dilution of the culture is continuous and the cell concentration kept constant, a steady state can be achieved; this is not readily achieved in monolayer culture. Maintenance of monolayer cultures is essentially cyclic with the result that growth rate varies depending on the phase of the growth cycle.

Monolayers are convenient for cytological and immunological observations, cloning, mitotic "shake off" (for cell synchronization of chromosome preparation) and *in situ* extractions without centrifugation.

TABLE 12.2. Properties of Monolayer and Suspension Cultures

	Monolayer	Suspension
Maintenance	Cyclic pattern of propagation (see text) Require dissociation Dependent on availability of substrate	Can be maintained at "steady state" Simple dilution at passage Dependent on medium volume only (with adequate gas exchange)
Results of differences in Geometry	Cell Interaction: metabolic cooperation, contact inhibition of movement and membrane activity, density limitation of growth Diffusion boundary effects (see text) Cell shape and cytoskeleton—spreading, motility, overlapping, underlapping	Homogeneous suspension Cell density limited by nutrient and hormonal concentration of the medium only Shearing effects in stirred cultures may damage some cells
Sampling and analysis	Good cytological preparations, chromosomes, immunofluorescence, histochemistry Enrichment of mitoses by "shake-off" (see Chapter 22) Serial extractions <i>in situ</i> possible without centrifugation	Bulk production of cells Ease of harvesting (no trypsinization required)
Which cells?	Most cell types except some hemopoietic cells and ascites tumors	Transformed cells and lymphoblastoid cell lines

At subculture a fragile or slowly growing line should be split 1:2; and a vigorous, rapidly growing line, 1:8 or 1:16. Once a cell line becomes continuous (usually taken as beyond 150 or 200 generations) the generation number is disregarded and the culture should simply be cut back to between 10^4 and 10^5 cells/ml. The split ratio or dilution is also chosen to establish a convenient subculture interval (perhaps 1 or 2 wk), and to ensure that the cells (1) are not diluted below that concentration which permits them to reenter the growth cycle with a lag period of 24 hr or less, and (2) do not enter plateau before the next subculture.

GROWTH CYCLE

Routine passage leads to the repetition of a standard growth cycle. It is essential to become familiar with this cycle for each cell line that is handled as this controls the seeding concentration, the duration of growth before subculture, the duration of experiments, and the appropriate times for sampling to give greatest consistency. Cells at different phases of the growth cycle behave differently with respect to proliferation, enzyme activity, glycolysis and respiration, synthesis of specialized products, and many other properties.

Outline

Set up a series of cultures at three different cell concentrations and count the cells at daily intervals until they "plateau."

Materials

Cell culture
24-well plates (sterile)
100 ml growth medium (sterile)
0.25% crude trypsin (for monolayer cultures only) (sterile)
plastic box to hold plates
CO₂ incubator or CO₂ supply to gas box

Protocol

1. Trypsinize cells as for regular subculture (see above)
2. Dilute cell suspension to 10^5 cells/ml, 3×10^4 cells/ml, and 10^4 cells/ml, in 25 ml medium for each concentration

3. Seed three 24-well plates, one at each cell concentration, with 1 ml per well. Add cell suspension slowly from the center of the well so that it does not swirl around the well. Similarly, do not shake the plate to mix the cells, as the circular movement of medium will concentrate the cells in the middle of the well

4. Place in a humid CO₂ incubator or sealed box gassed with CO₂

5. After 24 hr, remove plates from incubator and count the cells in three wells of each plate: (a) Remove medium completely from wells to be counted; (b) add 1 ml trypsin to each well; (3) incubate with trypsin; (4) after 15 min, disperse cells in trypsin and transfer 0.4 ml to counting fluid and count on cell counter

Note.

Hemocytometer counting may be used but may be difficult at lower cell concentrations. Reduce trypsin volume to 0.1 ml and disperse cells carefully without frothing using a micropipette and transfer to hemocytometer.

6. Return plate to incubator as soon as cell samples in trypsin are removed. The plate must be out of the incubator for the minimum length of time, to avoid disruption of normal growth

7. Repeat sampling at 48 and 72 hr as in steps 5 and 6

8. Change medium at 72 hr or sooner if indicated by pH drop (see above)

9. Continue sampling at daily intervals for rapidly growing cells (doubling time 12–14 hr) but reduce frequency of sampling to every 2 days for slowly growing cells (doubling time >24 hr), until plateau is reached

- 10.

Keep changing medium every 1, 2, or 3 days as indicated by pH

Analysis. 1. Calculate cell number per well, per ml of culture medium (same figure), and per cm² of available growth surface in well. (Stain one or two wells

(see Chapter 15) at each density to determine whether distribution of cells in wells is uniform and whether they grow up the sides of the well)

2. Plot cell density (per cm^2) and cell concentration (per ml), on a log scale, against time on a linear scale (Fig. 12.3)

3. Determine the lag time, population doubling time, and plateau density (see below and Fig 12.3)

4. Establish which is the appropriate starting density for routine passage. Repeat growth curve at intermediate cell concentrations if necessary

Variations. 1. Different culture vessels may be used, e.g., 25 cm^2 flasks, although more cells and medium will be required

2. Frequency of medium changing may be altered

3. Different media or supplements may be tested

Suspension cultures

1. Add cell suspension in growth medium to wells at a range of concentrations as for monolayer

2. Sample 0.4 ml at intervals as per trypsin samples.

Alternatively, seed two 75- cm^2 flasks with 20 ml for each cell concentration and sample 0.4 ml from each flask daily or as required. Mix well before sampling and keep flasks out of incubator for the minimum length of time. Do not feed cultures during growth curve

The growth cycle (Fig. 12.3) is conventionally divided into three phases.

The Lag Phase

This is the time following subculture and reseeding during which there is little evidence of an increase in cell number. It is a period of adaptation during which the cell replaces elements of the glycocalyx lost during trypsinization, attaches to the substrate, and spreads out. During spreading the cytoskeleton reappears and its reappearance is probably an integral part of the spreading process. Enzymes, such as DNA polymerase, increase, followed by the synthesis of new DNA and structural proteins. Some specialized cell products may disappear and not reappear until cessation of cell proliferation at high cell density.

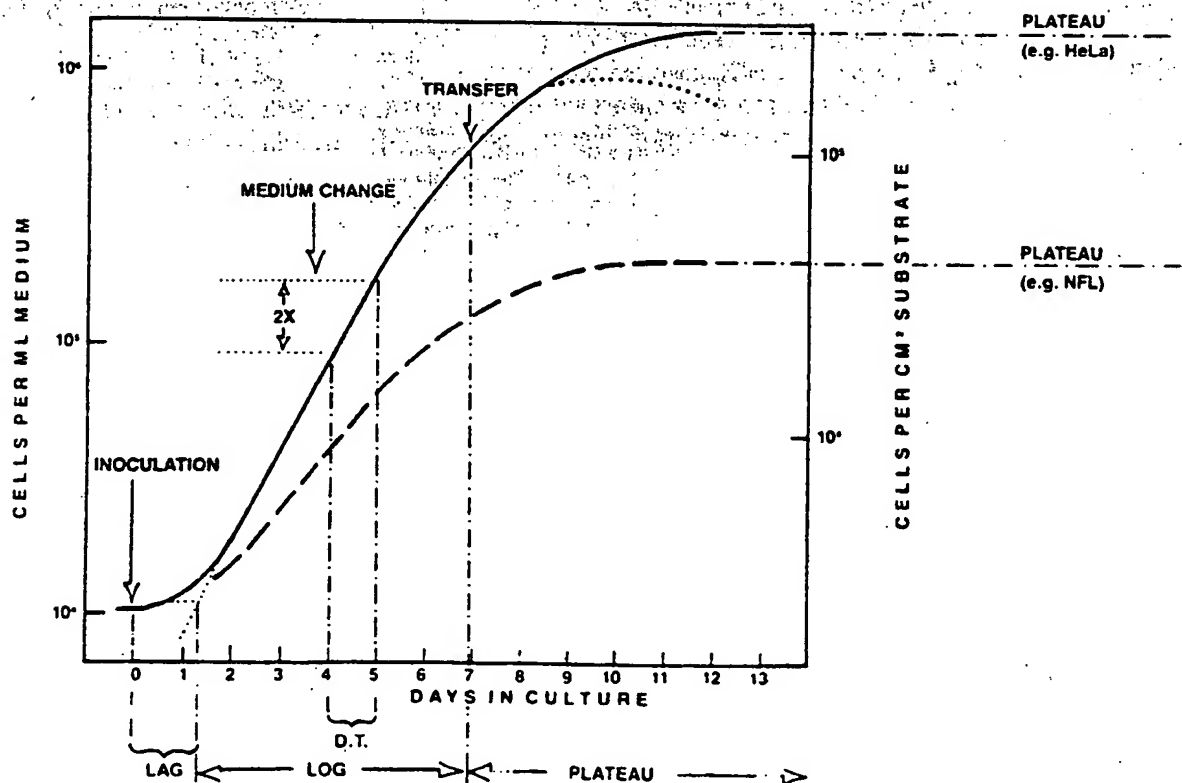


Fig. 12.3. Diagram of growth curve of a continuous cell line such as HeLa and a finite cell line, NFL (normal fetal lung fibroblasts).

The solid line represents growth of HeLa; the dashed line illustrates the lower plateau obtained with NFL (see text).

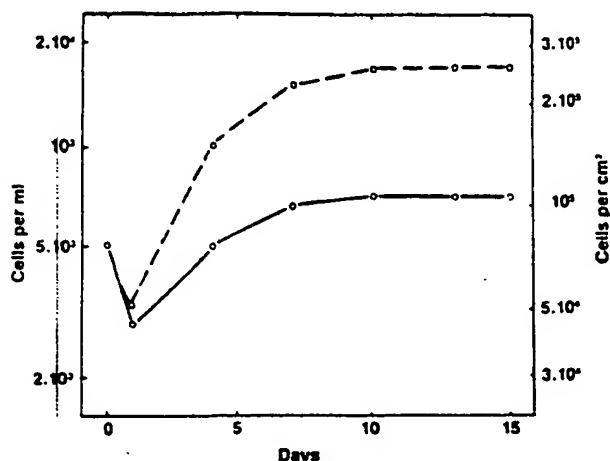


Fig. 12.4. Difference in plateaus (saturation densities) attained by cultures from normal brain (circles, solid line) and a glioma (squares, broken line). Cells were seeded onto 13-mm coverslips and 48 hr later the coverslips were transferred to 9-cm petri dishes with 20 ml growth medium, to minimize exhaustion of the medium.

The Log Phase

This is the period of exponential increase in cell number following the lag period and terminating one or two doublings after confluence is reached. The length of the log phase depends on the seeding density, the growth rate of the cells and the density at which cell proliferation is inhibited by density. In the log phase the growth fraction is high (usually 90–100%) and the culture is in its most reproducible form. It is the optimal time for sampling since the population is at its most uniform and viability is high. The cells are, however, randomly distributed in the cell cycle and, for some purposes, may need to be synchronized (see Chapter 22).

The Plateau Phase

Toward the end of the log phase, the culture becomes confluent—i.e., all the available growth surface is occupied and all the cells are in contact with surrounding cells. Following confluence the growth rate of the culture is reduced, and in some cases, cell proliferation ceases almost completely after one or two further population doublings. At this stage, the culture enters the plateau (or stationary) phase, and the growth fraction falls to between 0 and 10%. The cells may become less motile; some fibroblasts become oriented with respect to one another, forming a typical parallel array of cells. "Ruffling" of the plasma membrane is reduced, and the cell both occupies less surface area of substrate and presents less of its own surface to the medium. There may be a relative increase in the syn-

thesis of specialized versus structural proteins and the constitution and charge of the cell surface may be changed.

The phenomenon of cessation of motility, membrane ruffling, and growth was described originally by Abercrombie and Heaysman (1964) and designated "contact inhibition." It has since been realized that the reduction of the growth of normal cells after confluence is reached is not due solely to contact but may also involve reduced cell spreading [Stoker et al., 1968; Fölkman and Moscona, 1978] and depletion of nutrients, and, particularly, growth factors [Stoker, 1973; Dulbecco and Elkington, 1973; Westermark and Wasteson, 1975] in the medium [Holley et al., 1978]. The term "density limitation" of growth has, therefore, been used to remove the implication that cell-cell contact is the major limiting factor [Stoker and Rubin, 1967], and "contact inhibition" is best reserved for those events directly contingent on cell contact, i.e., reduced cell motility and membrane activity, resulting in the formation of a strict monolayer and orientation of the cells with respect to each other.

Cultures of normal epithelial and endothelial cells will stop growing after confluence and remain as a monolayer, implying that they are dependent on anchorage to the substrate for continued growth. (This may be analogous to anchorage to basement membrane *in vivo*.) Most cultures, however, with regular replenishment of medium, will continue to proliferate, although at a reduced rate, well beyond confluence, resulting in multilayers of cells. Human embryonic lung, or adult skin, fibroblasts, which express contact inhibition of movement, will continue to proliferate, laying down layers of collagen between the cell layers, until multilayers of six or more cells can be reached under optimal conditions [Kruse et al., 1970]. They still retain an ordered parallel array, however. The terms "plateau" and "stationary" are not strictly accurate, therefore, and should be used with caution.

Cultures which have transformed spontaneously or have been transformed by virus or chemical carcinogens will usually reach a higher cell density in the plateau phase than their normal counterparts [Westermark, 1974] (Fig. 12.4). This is accompanied by a higher growth fraction and loss of density limitation. These cultures are often *anchorage independent* for growth—i.e., they can easily be made to grow in suspension (see Density Limitation of Growth in Chapter 16; also see Chapter 2).

The construction of a growth curve from cell counts performed at intervals after subculture enables the measurement of a number of parameters which should

be found to be characteristic of the cell line under a given set of culture conditions. The first of these is the duration of the *lag period* or "lag time" obtained by extrapolating a line drawn through the points on the exponential phase until it intersects the seeding or inoculum concentration (see Fig. 12.3), and reading off the elapsed time since seeding equivalent to that intercept. The second is the *doubling time*, i.e., the time taken for the culture to increase two-fold in the middle of the exponential, or "log", phase of growth. This should not be confused with the *generation time* or *cell cycle time* (see Chapter 19), which are determined by measuring the transit of a population of cells through the cell cycle until they return to the same point in the cell cycle.

The last of the commonly derived measurements from the growth cycle is the "*plateau level*" or "*saturation density*." This is the cell concentration in the plateau phase and is dependent on cell type and frequency of medium replenishment. It is difficult to measure accurately as a steady state is not achieved as easily as in the log phase. Ideally the culture should be perfused, but a reasonable compromise may be achieved by growing the cells on a restricted area, say a small-diameter coverslip (15 mm) in a large-diameter petri dish (90 mm); with 20 ml of medium replaced daily (see Chapter 16). Under these conditions, medium limitation of growth is minimal, and cell density exerts the major effect. Counting the cells under these conditions gives a more accurate and reproducible measurement. "Plateau" does not imply complete cessation of cell proliferation but represents a steady state where cell division is balanced by cell loss.

The maximum cell concentration in suspension cultures, which are not limited by available substrate, is usually limited by available nutrients. By fortifying the medium with a higher concentration of amino acids, Pirt and others [Birch and Pirt, 1971; Blaker et al., 1971] were able to obtain a maximum cell concentration of 5×10^6 cells/ml for L"S" cells, far in excess of what can be achieved with attached cells.

SLOW CELL GROWTH

Even in the best-run laboratories, problems may arise in routine cell maintenance. Some may be attributed to microbiological contamination (see Chapter 17), but often the cause lies in one or more alterations in culture conditions. The following check list may help to track these down:

1. Any change in procedure or equipment?
2. Medium:

- a. Medium adequate?—check against other media (see Chapter 9).
- b. Frequency of changing adequate?
- c. pH: Check that it is within 7.0–7.4 during culture.
- d. Osmolality: check on osmometer.
- e. Component missed out: make up fresh batch.
- f. New batch of stock medium which is faulty?
- g. If BSS-based, is BSS satisfactory? (Check with other users.)
- h. If water-based, is water satisfactory (check with other users, or against fresh IX medium, bought in).
- i. Check still—deionizer—conductivity, contamination—glass boiler—residue.
- j. Storage vessel, for algal or fungal contamination: chemical traces in plastic.
- k. HCO_3^- .
- l. Antibiotics.

3. Serum:

- a. New batch? Check supplier's quality control.
- b. Check concentration. Too low or too high?
- c. Reconfirm lack of toxicity, growth promotion, and plating efficiency.

4. Glassware or plastics:

- a. If new, check against previous stock.
- b. Wash-up—other cells showing symptoms? Other users having trouble?
- c. Trace contamination of glass? Check growth on plastic.

5. Cells: If other people's cells are all right:

- a. Contamination (see also Chapter 17):
 - i) Bacterial, fungal—grow up without antibiotics.
 - ii) Mycoplasma:
 - (A) Stain Culture with Hoechst 33258 (Chapter 17).
 - (B) Check for cytoplasmic DNA (incorporation of radioactive thymidine) by autoradiography.
 - (C) Get commercial test done (e.g., Flow Laboratories or Microbiological Associates).
 - iii) Viral—difficult to detect—try E M or fluorescent antibody.

- b. Seeding density too low at transfer.
 - c. Transferred too frequently.
 - d. Allowed to remain for too long in plateau before transfer.
6. Hot room and incubators: check temperature and stability.

Chapter 13 Cloning and Selection of Specific Cell Types

It can be seen from the preceding two chapters that a major recurrent problem in tissue culture is the preservation of a specific cell type and its specialized properties. While environmental conditions undoubtedly play a significant role in maintaining the differentiated properties of a culture, the selective overgrowth of unspecialized cells is still a major problem.

CLONING

The traditional microbiological approach to the problem of culture heterogeneity is to isolate pure cell strains by cloning, but the success of this technique in animal cell culture is limited by the poor cloning efficiencies of most primary cultures.

A further problem of cultures derived from normal tissue is that they may only survive for a limited number of generations (see Chapter 2), and by the time a clone has produced a usable number of cells, it may already be near to senescence (Fig. 13.1). Even cultures which do not die out may have undergone alteration by the time the cloned cell line is established. Cloning is most successful in isolating variants from continuous cell lines, but even then considerable heterogeneity may arise within the clone as it is grown up for use (See Chapter 18).

Coon and Cahn [1966] were able to clone cartilage- and pigment-producing cell strains. Under the correct conditions, these cultures were able to retain their specialized functions over many generations. Similarly, Clark and Pateman [1978] isolated a Kupffer cell line from Chinese hamster liver by cloning the primary culture.

Cloning has also been used to isolate specific biochemical mutants and cell strains with marker chromosomes and may help to reduce the heterogeneity of a culture.

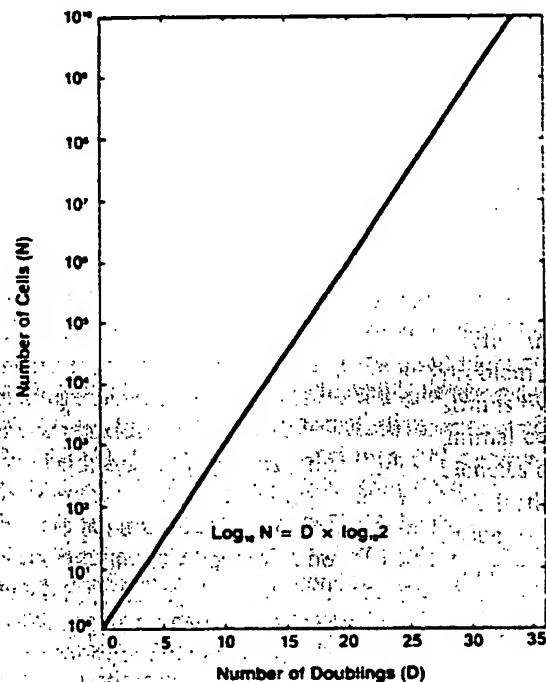


Fig. 13.1. Relationship of cell yield in a clone to the number of population doublings—e.g., 20 doublings are required to produce 10^6 cells.

Dilution Cloning [Puck and Marcus, 1955]

Outline

Seed cells at low density, incubate until colonies form, isolate and propagate into cell strain (Fig. 13.2).

Materials

pipettes
medium
trypsin

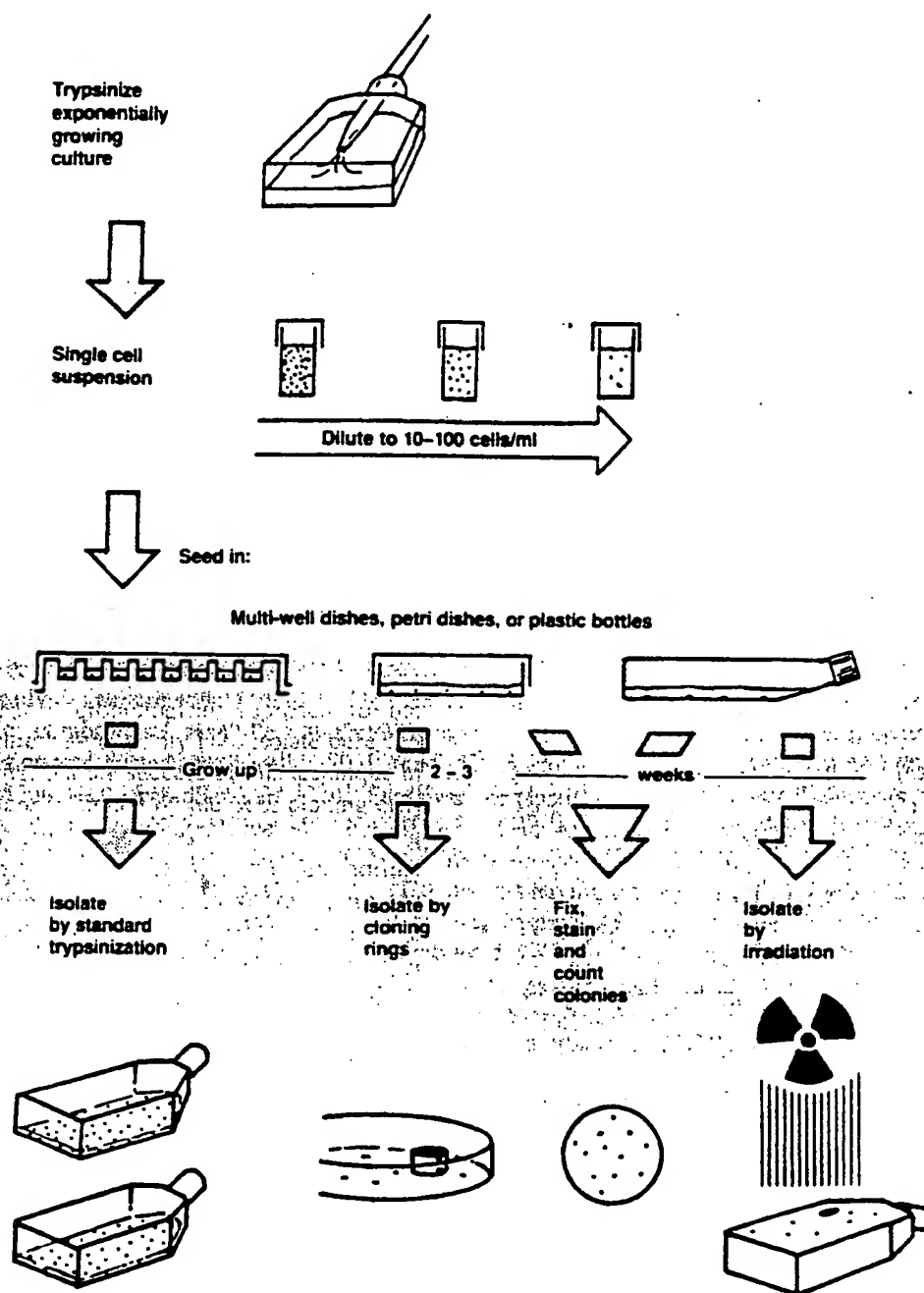


Fig. 13.2. Cloning cells in monolayer culture. When clones form, they may be (1) isolated (a) directly, from multi-well dishes, (b) by the cloning ring technique (center left of figure), (c) by irradi-

diating the flask while shielding one colony (bottom right-hand side of figure), or (2) fixed, stained, and counted (center right of figure) for analysis.

culture flasks or dishes
tubes for dilution
hemocytometer or cell counter

Protocol

1. Trypsinize cells (see Chapter 12) to produce a single cell suspension. Undertrypsinizing will produce clumps; overtrypsinizing will reduce viability

2. While cells are trypsinizing, number flasks or dishes and measure out medium for dilution steps (four dilution steps may be necessary to reduce a regular monolayer to a concentration suitable for cloning)

3. When cells round up and start to detach, disperse the monolayer in medium containing serum or trypsin inhibitor, count and dilute to desired seeding concentration. If cloning the cells for the first time, choose a range of 10, 50, 100 and 200 cells/ml (Table 13.1)

4. Seed petri dishes or flasks with the requisite amount of medium (see Chapter 12), place petri dishes in a humid CO₂ incubator or gassed sealed container (2–10% CO₂, see Chapter 9), or gas flasks with CO₂, seal with cap, and place in dry incubator

5. Leave untouched for 1 wk. If colonies have formed, isolate (see below); if not, replace medium and continue to culture for a further week, or feed again and culture for a further week, or feed again and culture for 3 wk if necessary. If no colonies have appeared by 3 wk, it is unlikely that they will do so

Stimulation of Plating Efficiency

When cells are plated at low densities, the survival falls in all but a few cell lines. This does not usually present a severe problem with continuous cell lines where the plating efficiency seldom drops below 10%, but with primary cultures and finite cell lines, the plating efficiency may be quite low—0.5%–5% or even zero. Numerous attempts have been made to improve plating efficiencies, based on the assumption

either that cells require a greater range of nutrients at low densities or that cell-derived diffusible signals or conditioning factors are present in high-density cultures and absent or too dilute at low densities. The intracellular metabolic pool of a leaky cell in a dense population will soon reach equilibrium with the surrounding medium, while that of an isolated cell never will. This was the basis of the capillary technique of Sandford et al. [1948], when the L929 clone of L-cells was first produced. The confines of the capillary tube allowed the cell to create a locally enriched environment mimicking the higher cell density state. In microdrop techniques developed later, the cells were seeded as a microdrop under liquid paraffin. Keeping one colony separate from another, as in the capillary techniques, colonies could be isolated subsequently. As media improved, however, plating efficiencies increased, and Puck and Marcus [1955] were able to show that cloning cells by simple dilution (as described above) in association with a feeder layer of irradiated mouse embryo fibroblasts (see below) gave acceptable cloning efficiencies, although subsequent isolation required trypsinization from within a collar placed over each colony.

Some modifications which may improve clonal growth are listed below.

Medium. Choose a rich medium such as Ham's F12 or one which has been optimized for the cell type in use, e.g., MCDB 105 [Ham and McKeehan, 1978] for human fibroblasts, Ham's F12 or MCDB 301 for CHO [Ham 1963; Hamilton and Ham, 1977].

Serum. Where serum is required, fetal bovine is generally better than calf or horse. Select a batch for cloning experiments which gives a high plating efficiency during tests.

Conditioning. (1) Grow homologous cells, embryo fibroblasts, or another cell line to 50% of confluence, change to fresh medium, incubate for a further 48 hr, and collect the medium. (2) Filter through a 0.2- μ m sterilizing filter (the medium may need to be clarified first by centrifugation 10,000 g, 20 min, or filtration through 5- μ m and 1.2- μ m filters (see Chapter 10, section on sterilization of serum). (3) Add to cloning medium 1 part conditioned medium to 2 parts cloning medium.

Feeder layers (Fig. 13.3, regular feeder layer). (1) Trypsinize embryo fibroblasts from primary culture (see Chapter 11) and reseed at 10⁵ cells/ml. (2) At 50% confluence, add mitomycin-C, 2 μ g/10⁶ cells, 0.25 μ g/ml, overnight [MacPherson and Bryden, 1971], or ir-

TABLE 13.1. Relationship of Seeding Density to Plating Efficiency

Expected plating efficiency	Optimal cell number to be seeded	
	Per ml	Per cm ²
0.1%	10 ⁴	2 × 10 ⁴
1.0%	10 ³	200
10%	100	20
50%	20	4
100%	10	2

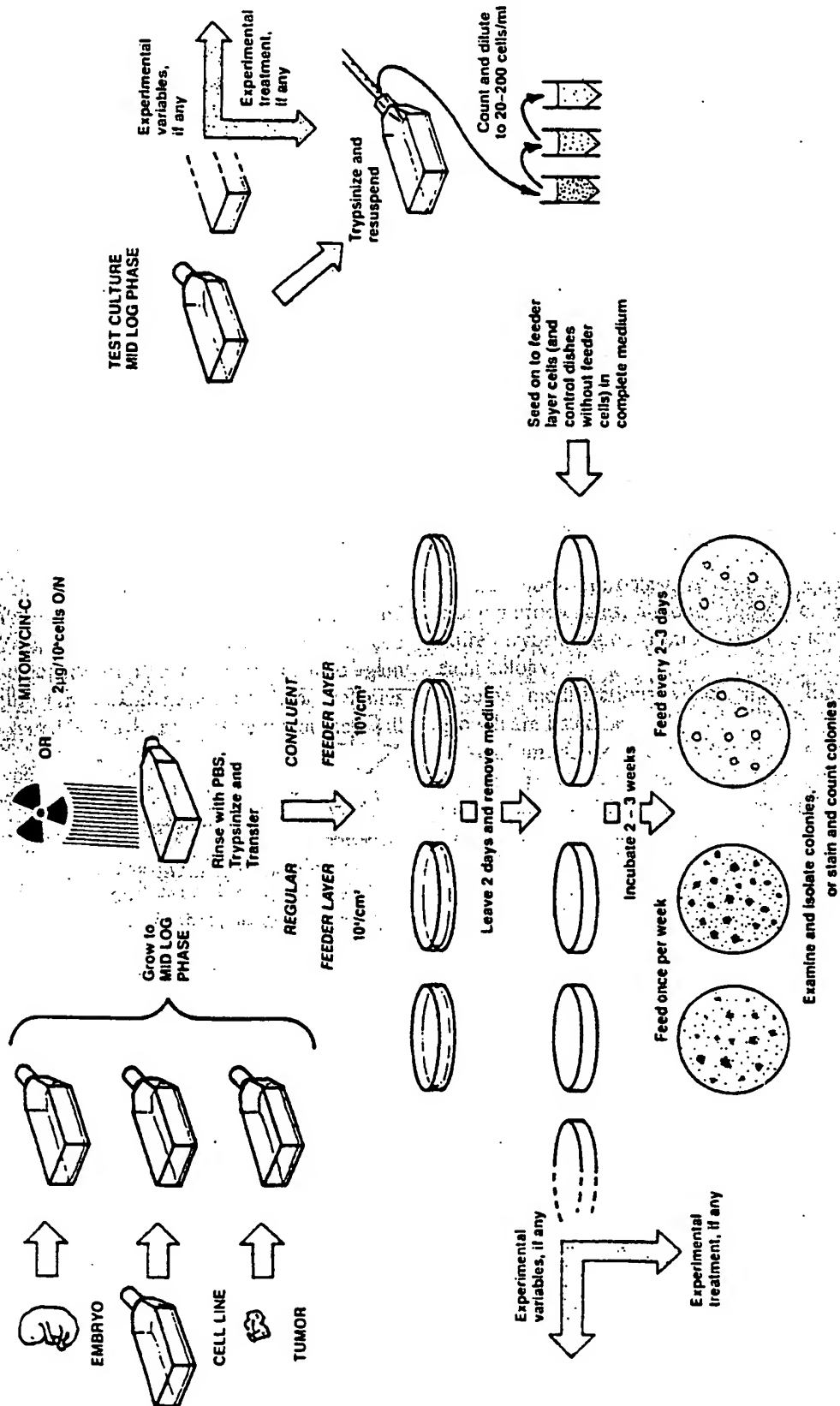


Fig. 13.3. Cloning cells in the presence of a feeder layer. A low-density feeder layer ($\sim 10^4$ cells/cm²) (left-hand side of figure) is used to enhance cloning efficiency and fibroblastic over growth (see also Fig. 13.8 and text). chond growth, while a high-density, confluent, feeder layer (e.g., 2×10^6 normal human

radiate culture with 3,000 rad. (3) Change the medium after treatment, and after a further 24 hr, trypsinize the cells and reseed in fresh medium at 5×10^4 cells/ml (10^4 cells/cm²). (4) Incubate for a further 24–48 hr and then seed cells for cloning. The feeder cells will remain viable for up to 3 wk but will eventually die out and are not carried over if the colonies are isolated.

Other cell lines or homologous cells may be used to improve the plating efficiency but heterologous cells have the advantage that if clones are to be isolated later, chromosome analysis will rule out accidental contamination from the feeder layer.

Hormones. Insulin, 1–10 IU/ml has been found to increase the plating efficiency of several cell types [Hamilton and Ham, 1977]. Dexamethasone, 2.5×10^{-5} M, ~ 10 μ g/ml (a soluble synthetic hydrocortisone analogue) improves the plating efficiency of human normal glia, glioma, fibroblasts, and melanoma, and chick myoblasts, and will give increased clonal growth (colony size) if removed 5 d after plating [Freshney et al., 1980a,b].

Intermediary metabolites. Keto acids, e.g., pyruvate or α -ketoglutarate [Griffiths and Pirt, 1967; McKeehan and McKeehan, 1979] and nucleosides [α -medium, Stanners et al., 1971], have been used to supplement media and are already included in the formulation of a rich medium like Ham's F12. Pyruvate is also added to Dulbecco's modification of Eagle's MEM [Dulbecco and Freeman, 1959; Morton, 1970].

Carbon dioxide. CO₂ is essential to obtain maximum cloning efficiency for most cells. While 5% is most usual, 2% is sufficient for many cells, and may even be slightly better for human glia and fibroblasts. HEPES (20 mM) may be used with 2% CO₂, protecting the cells against pH fluctuations during feeding and in the event of failure of the CO₂ supply. (Using 2% CO₂ also cuts down in the consumption of CO₂.) At the other extreme, Dulbecco's modification of Eagle's MEM is normally equilibrated with 10% CO₂ and is frequently used for cloning myeloma hybrids for monoclonal antibody production. The concentration of bicarbonate must be adjusted if the CO₂ tension is altered so that equilibrium is reached at pH 7.4 (see Table 8.2).

Treatment of substrate. Polylysine improves the plating efficiency of human fibroblasts in low serum concentrations [McKeehan and Ham, 1976] (see Chapter 8). (1) Add 1 mg/ml poly-D-lysine in water to plates (~ 5 ml/25 cm²). (2) Remove and wash plates with 5 ml PBSA per 25 cm². The plates may be used immediately or stored for several weeks before used.

Fibronectin also improves the plating of many cells [Barnes and Sato, 1980]. The plates should be pre-treated with 5 μ g/ml fibronectin incorporated in the medium.

Trypsin. Pure, twice recrystallized, trypsin used at 0.05 μ g/ml may be preferable to crude trypsin, but there are conflicting reports on this. McKeehan [1977] noted a marked improvement in plating efficiency when trypsinization (pure trypsin) was carried out at 4° C.

Multiwell Dishes

If clones are to be isolated, cloning by dilution directly into microwells (microtitration dishes or 24-well plates, see Fig. 8.4) makes subsequent harvesting easier. The plates must be checked regularly after seeding, however, to confirm that either only one cell is present per well at the start or, if there is more than one cell per well, they are not clumped and that only one cell gives rise to a colony, i.e., that the colonies which form are truly clonal in origin, and only one colony forms in the well.

Semisolid Media

Some cells, particularly virally transformed fibroblasts, will clone readily in suspension. To hold the colony together and prevent mixing, the cells are suspended in agar or methocel and plated out over an agar underlay or into nontissue culture grade dishes.

Cloning in agar. See Figure 13.4 and see also Metcalf [1970], Pike and Robinson [1970], and MacPherson [1973].

Outline

Agar is liquid at high temperatures but is a gel at 36.5° C. Cells are suspended in warm agar, and, when incubated after the agar gels, will form discrete colonies which may be isolated easily.

Materials

2% agar
medium, e.g., Ham's F12, RPMI 1640,
or CMRL 1066
fetal bovine serum
pipettes
35-mm petri dishes
50-mm \times 8-mm test tubes
boiling water bath
water bath at 45° C
ice tray

Note. preparing medium and cells, work out cell dilutions and label petri dishes or multiwell plates.

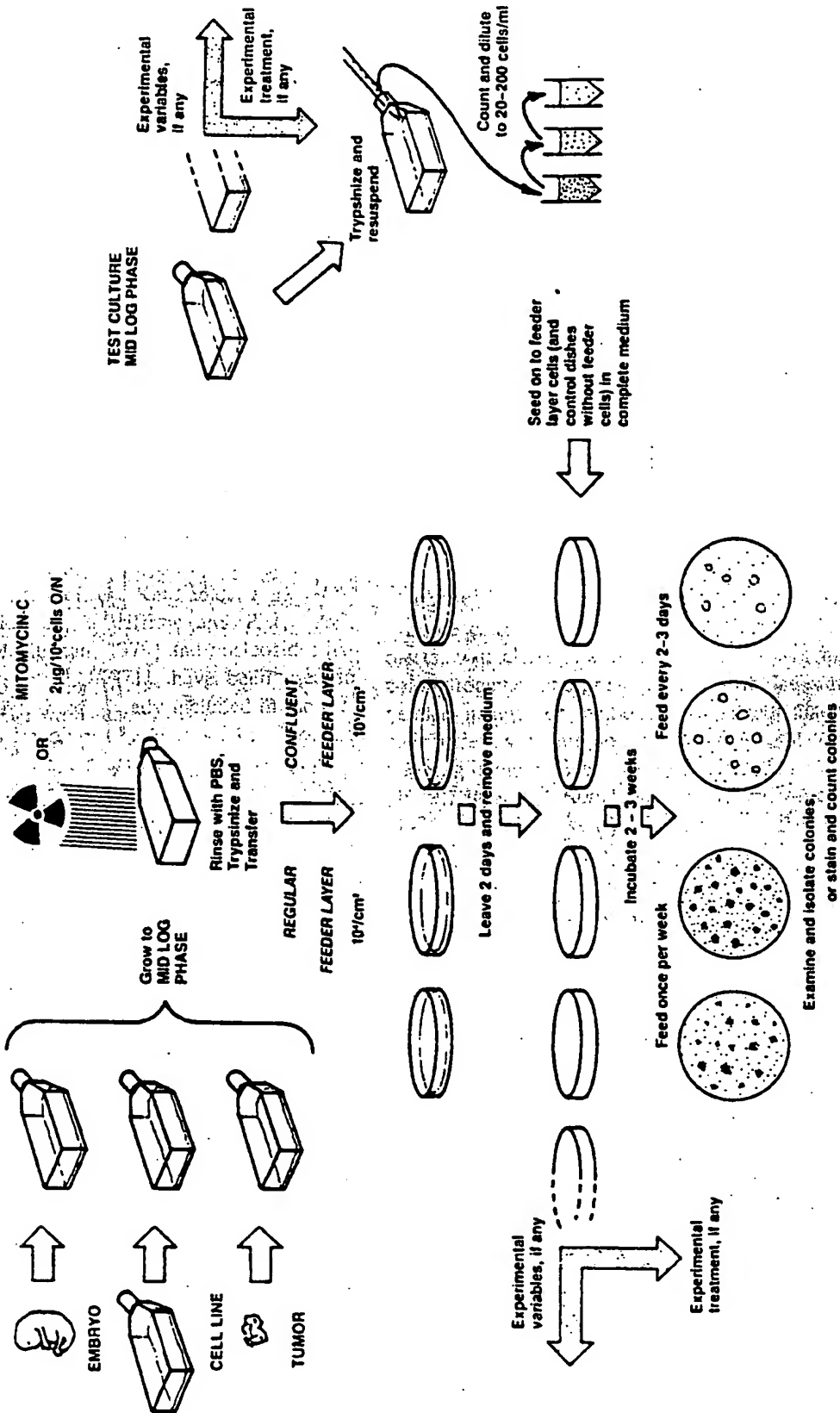


Fig. 13.3. Cloning cells in the presence of a feeder layer. A low-density feeder layer (diploid fibroblasts or glial cells/cm²) can be used as a selective substrate to minimize ($\sim 10^4$ cells/cm²) (left-hand side of figure) is used to enhance cloning efficiency and fibroblastic over growth (see also Fig. 13.8 and text). clonal growth, while a high-density, confluent, feeder layer (e.g. 2×10^5 normal human

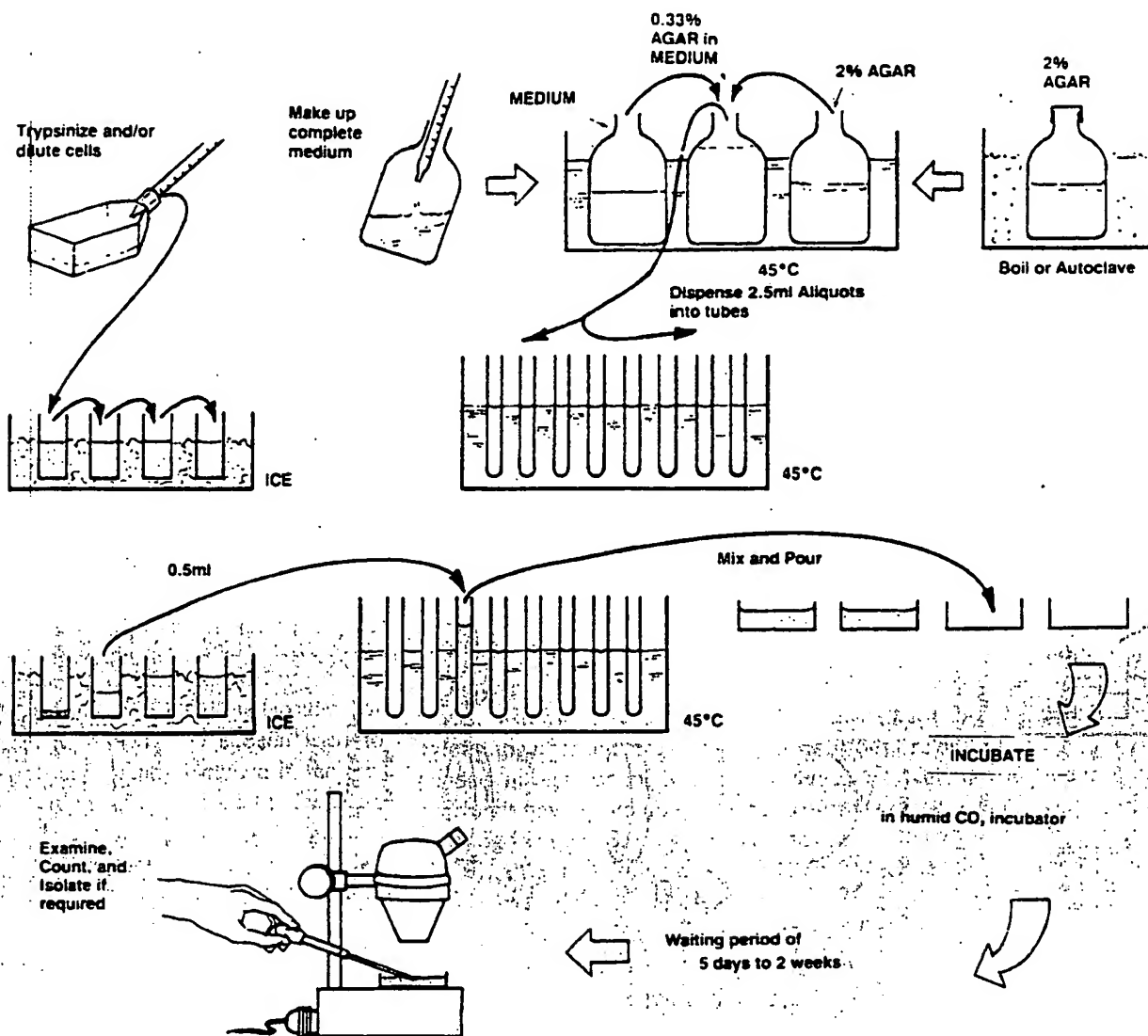


Fig. 13.4. Cloning cells in suspension in agar.

Convenient cell numbers per 35-mm dish are 1,000, 333, 111, 37.

Protocol

1. Dissolve agar by placing bottle in a boiling water bath or autoclave. Cool to 80° C and transfer to 45° C water bath. (If you are delayed, keep agar at 60° C)
2. Make up medium A: 70 ml medium and 30 ml FBS, keep at 45° C
3. Make up medium B: 60 ml medium A and 12 ml

2% agar, keep at 45° C

4. Count the cell suspension and dilute to give 2,000/ml, 667/ml (1:3), 222/ml (1:3), 74/ml (1:3). Place on ice
5. Place 5-ml test tubes in rack and keep at 45° C
6. Add 2.5 ml medium B to test tubes
7. Add 0.5-ml cell suspension to one tube, mix, and pour into dish immediately
8. After all dishes have been poured, place them at

4° C for 10 min

9.

Before incubating the dishes in a humid CO₂ incubator, it is advisable to put them into another container to try to avoid contamination of the cultures from the moist atmosphere of the incubator. (a)

Place petri dishes or multiwell plates in a plastic box with a lid and containing a dish of water. (The box should be washed first with 70% alcohol and allowed to dry.) (b) When using 35-mm petri dishes, two can be put into a 10-cm petri dish (nontissue culture grade) with a third 35-mm petri

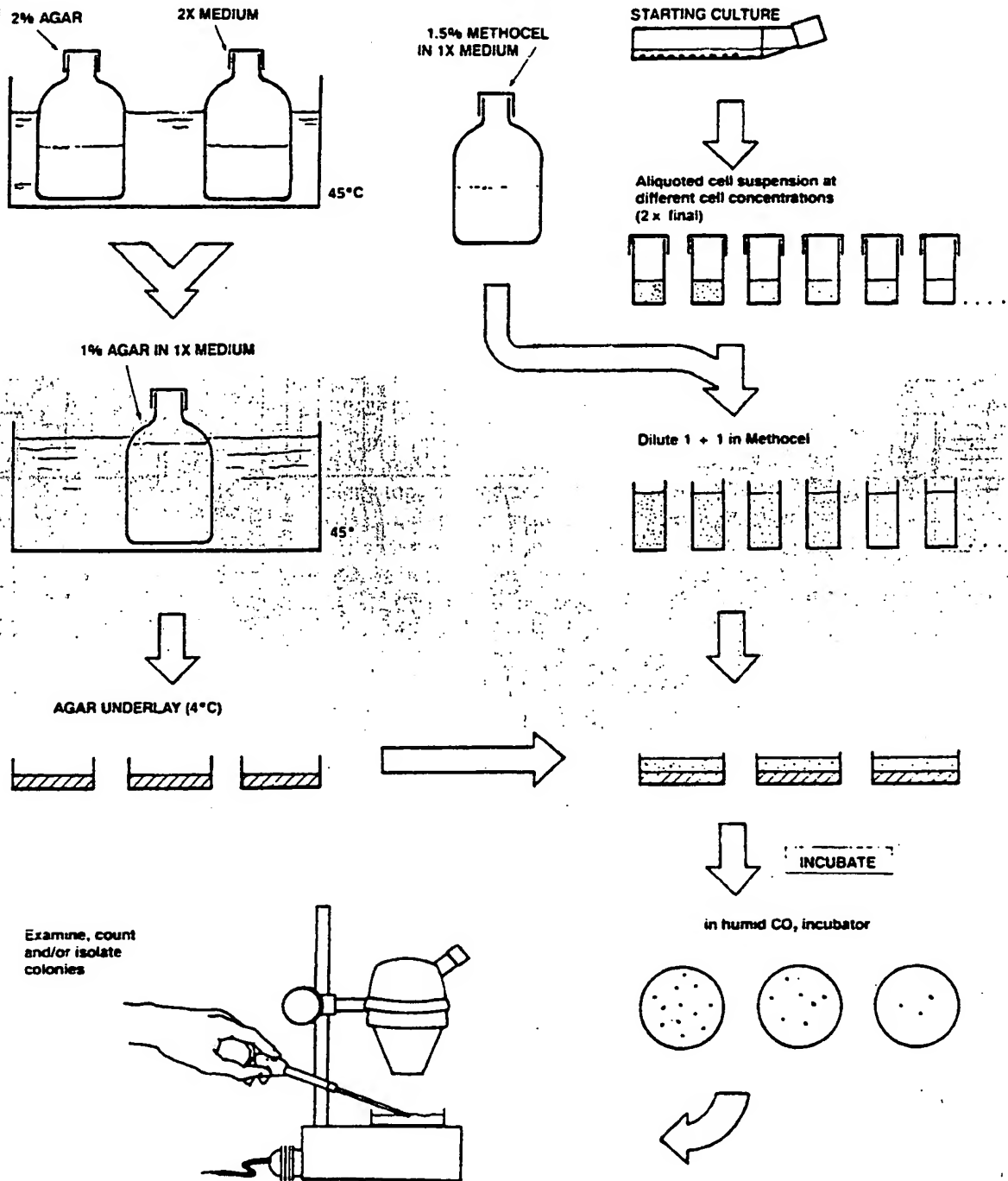


Fig. 13.5. Cloning cells in suspension in Methocel over an agar underlay.

dish containing 3 ml sterile water

Note. Rinse all pipettes used for agar with hot water before discarding.

Cloning in methocel over agar base [Buick et al., 1979].

Outline

Suspend cells in medium containing Methocel and seed into dishes containing gelled agar medium (Fig. 13.5).

Materials

As for agar cloning; 1.36% Methocel (4,000 counts/sec) in deionized distilled water.

Protocol

1. Prepare agar underlay by heating sterile 1.0–2.0% agar to 100° C, bring to 45° C, and dilute with an equal volume of double-strength medium at 45° C (prepare from $\times 10$ concentrate to half the recommended final volume and add twice the normal concentration of serum). Plate out 1 ml immediately into 35-mm dishes or 6 \times 35 mm multiwell plates, and allow to gel at 4° C for 10 min.
2. Trypsinize or collect cells from suspension and dilute to double the required final concentration.
3. Dilute the cells with an equal volume of methocel and plate out 1 ml over the agar underlay (10–1,000 cells per dish for continuous cell strains but up to 5×10^5 per dish may be needed for primary cultures).
4. Incubate until colonies form. Since the colonies form at the interface between the agar and the Methocel, fresh medium may be added, 1 ml per dish or well, after 1 wk and removed and replaced with more fresh medium after 2 wk without disturbing the colonies.

Many of the recommendations applying to medium supplementation for monolayer cloning also apply to suspension cloning. In addition, sulphydryl compounds such as mercaptoethanol (5×10^{-5} M), glutathione (1 mM), or α -thioglycerol (7.5×10^{-5} M) [Iscoe et al., 1980] are sometimes used. Macpherson

[1973] found the inclusion of DEAE dextran was beneficial for cloning.

Most cell types clone in suspension with a lower efficiency than in monolayer, some cells by two or three orders of magnitude. Isolation of colonies is, however, much easier.

Isolation of Clones

Monolayer clones—multiwell plates. If cells are cloned directly into multiwell plates (see above), colonies may be isolated by trypsinizing individual wells. It is necessary to confirm the clonal origin of the colony during its formation by regular microscopic observation.

Cloning rings. If cloning is performed in petri dishes, there is no physical separation between colonies. This must be created by removing the medium and placing a ring around the colony to be isolated (Fig. 13.6).

Outline

The colony is trypsinized from within a porcelain, Teflon, or stainless steel ring and transferred to one of the wells of a 24- or 12-well plate, or directly to a 25-cm² flask (see step 3 above) (Fig. 13.7).

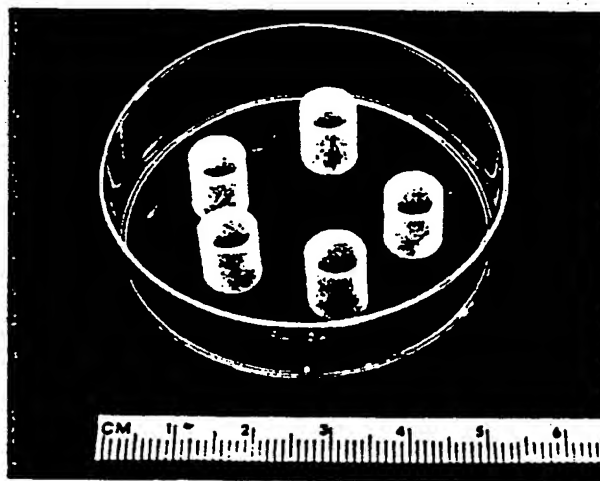


Fig. 13.6. Cloning rings. Porcelain rings (Fisher) are illustrated, but thick-walled stainless steel rings (e.g., roller bearings) or plastic (e.g., cut from nylon or Teflon thick-walled tubing) can be used. Whatever the material, the base must be smooth, to seal with silicone grease onto the base of the petri dish, and the internal diameter just wide enough to enclose one whole clone, without overlapping adjacent clones.

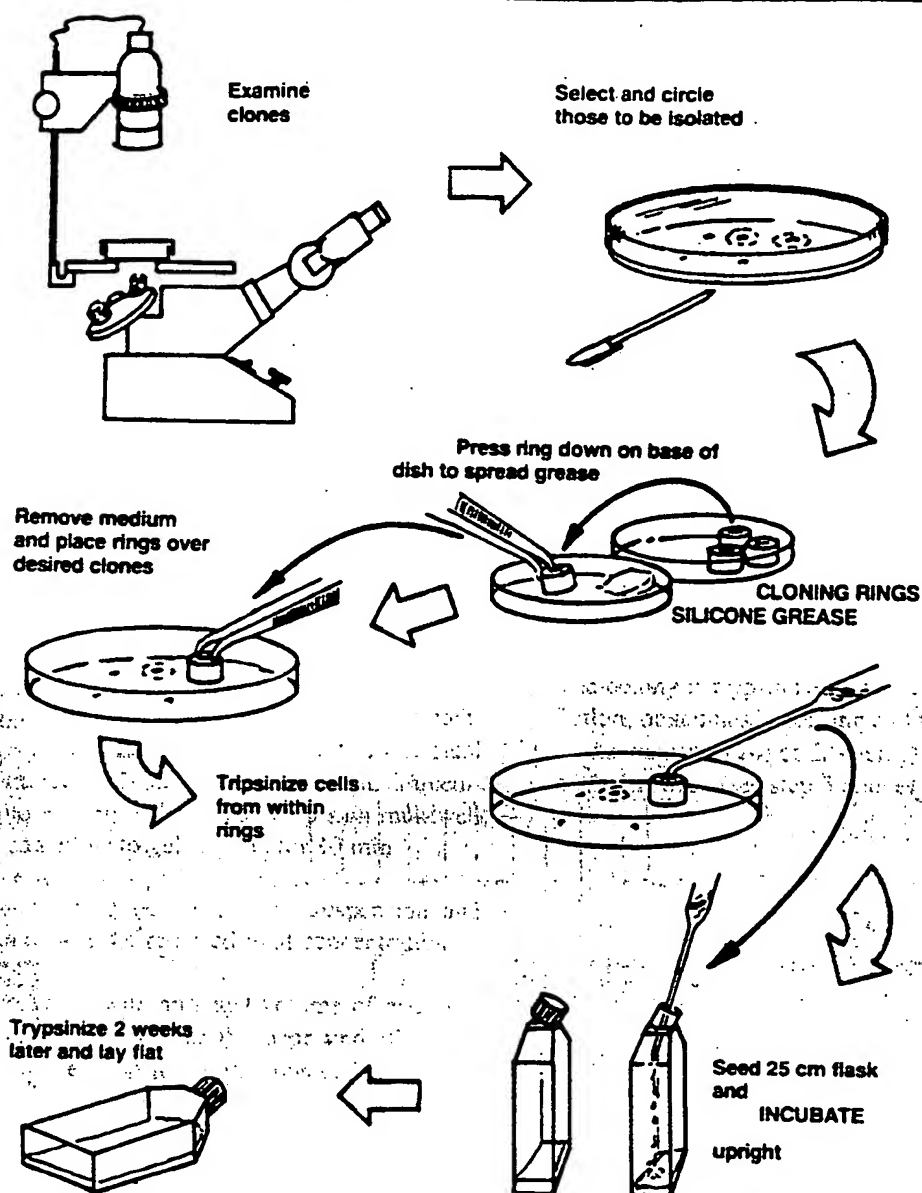


Fig. 13.7. Isolation of clones with cloning rings.

Materials

cloning rings
silicone grease
Pasteur pipettes with bent end
0.25% trypsin
medium
24-well plate and/or 25 cm² flasks
sterile forceps

Protocol

1. Sterilize cloning rings and silicone grease separately in glass petri dishes, by dry heat
2. Prepare about 20 bent Pasteur pipettes by heating briefly in a bunsen flame and allowing about 12 mm of the tip to drop under gravity. If the pipette

is held at 30° above horizontal, the bend will be 120°. Place pipettes in sterile test tubes and allow to cool before use

3.

Examine clones and mark those that you wish to isolate with a felt tip marker on the underside of the dish

4.

Remove medium from dish

5.

Using sterile forceps, take one cloning ring, dip in silicone grease, and press down on dish alongside silicone grease to spread the grease round the base of the ring

6.

Place ring around desired colony

7.

Repeat steps 5 and 6 for two or three other colonies in same dish

8.

Add sufficient 0.25% trypsin to fill the hole in ring (~0.4 ml), leave 20 sec, and remove

9.

Close dish and incubate for 15 min

10.

Add 0.4 ml medium to each ring

11.

Taking each clone in turn, pipette medium up and down to disperse cells, and transfer to a well of a 24-well plate, or to a 25-cm² flask standing on end. Use a separate pipette for each clone

12.

Wash out ring with a second 0.4 ml medium, and transfer to same well

13.

Close plate and incubate, or if using flasks, add 1 ml medium and incubate standing on end

14.

When clone grows to fill well, transfer up to 25-cm² flask, incubated conventionally with 5 ml me-

dium. If using up-ended flask technique, remove medium when end of flask confluent, trypsinize cells, resuspend in 5 ml medium, and lay flask down flat. Continue incubation

The cloning ring technique may be applied when cells are cloned in a plastic flask by swabbing the flask with alcohol and slicing the top off with a heated sterile scalpel or hot wire. Thereafter proceed as for petri dishes.

Irradiation. Alternatively, where an irradiation source is available, clones may be isolated by shielding one and irradiating the rest of the monolayer.

Outline

Invert the flask under an x-ray machine or ⁶⁰Co source, screening the desired colony with lead.

Materials

x-ray or cobalt source
piece of lead 2 mm thick
PBSA
0.25% trypsin
medium

Protocol

1. Select desired colony
2. Invert flask under x-ray or cobalt source
3. Cover colony with a piece of lead 2 mm thick
4. Irradiate with 3,000 rad
5. Return to sterile area and remove medium, trypsinize, and allow cells to reestablish in the same bottle, using the irradiated cells as a feeder layer

Fig. 13.8. Selective cloning of breast epithelium on a confluent feeder layer. *a.* Colonies forming on plastic alone after seeding 4,000 cells from a breast carcinoma culture/cm² (2×10^4 cells/ml). Small dense colonies are epithelial cells, larger stellate colonies are fibroblasts. *b.* Colonies of cells from the same culture, seeded at 400 cells/cm² (2,000 cells/ml) on a confluent feeder layer of FHS 74 Int cells [Owens et al., 1974]. The epithelial colonies are much larger than in *a.*, the plating efficiency is higher, and there are no fibroblastic colonies. *c.* Colonies from a different breast carcinoma culture plated onto the same feeder layer. Note different colony morphology with lighter stained cen-

ter and ring at point of interaction with feeder layer. *d.* Colonies from normal breast culture seeded onto FHI cells (fetal human intestine similar to FHS 74 Int). There are a few small fibroblastic colonies present in *c* and *d* (after a technique described by Dr. A.J. Hackett, personal communication).

Fig. 13.9. Selective growth of glioma on confluent feeder layer. Cells were seeded at 2×10^4 /ml (4×10^4 /cm²) onto confluent, mitomycin-C treated feeder layers (see text) of FHS 74 Int cells [Owens et al., 1974] and labeled at intervals thereafter with ³H-thymidine (see text), extracted, and counted.

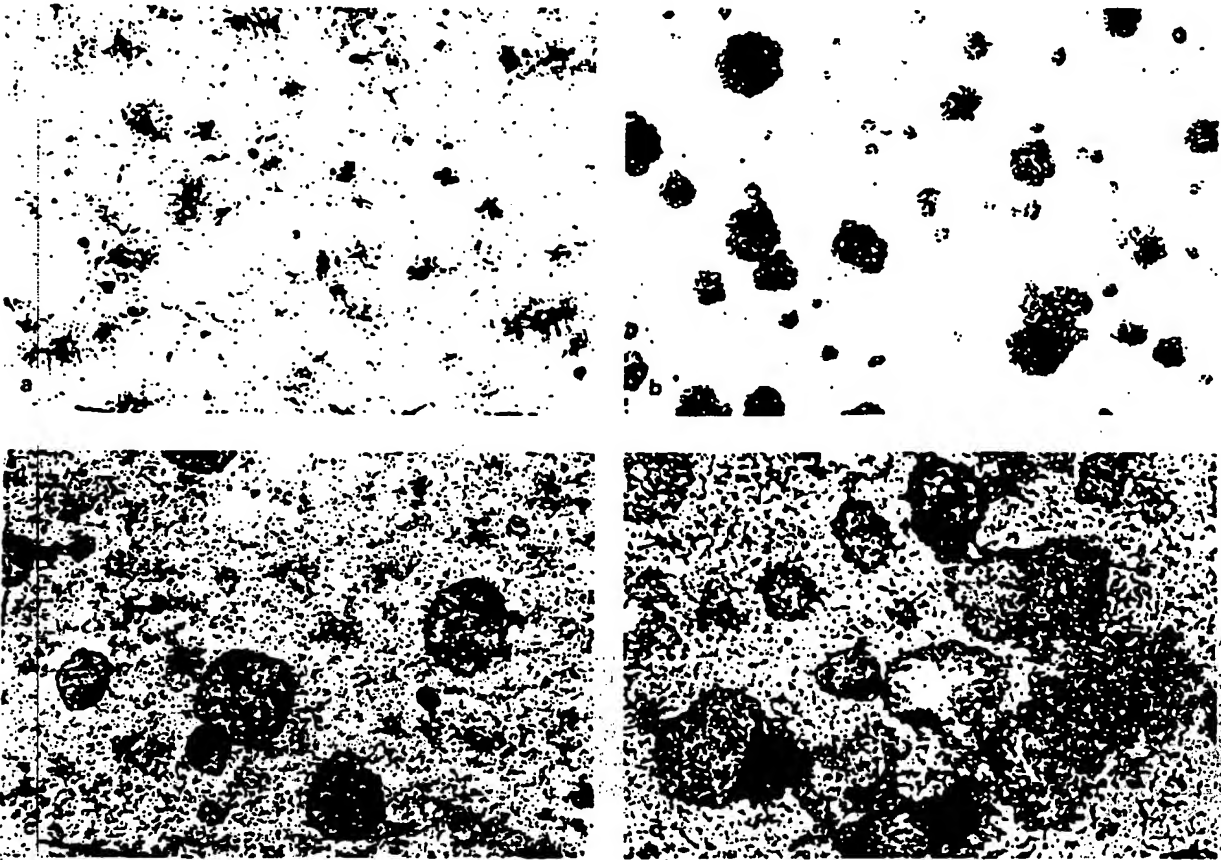


Figure 13.8. Legend on facing page.

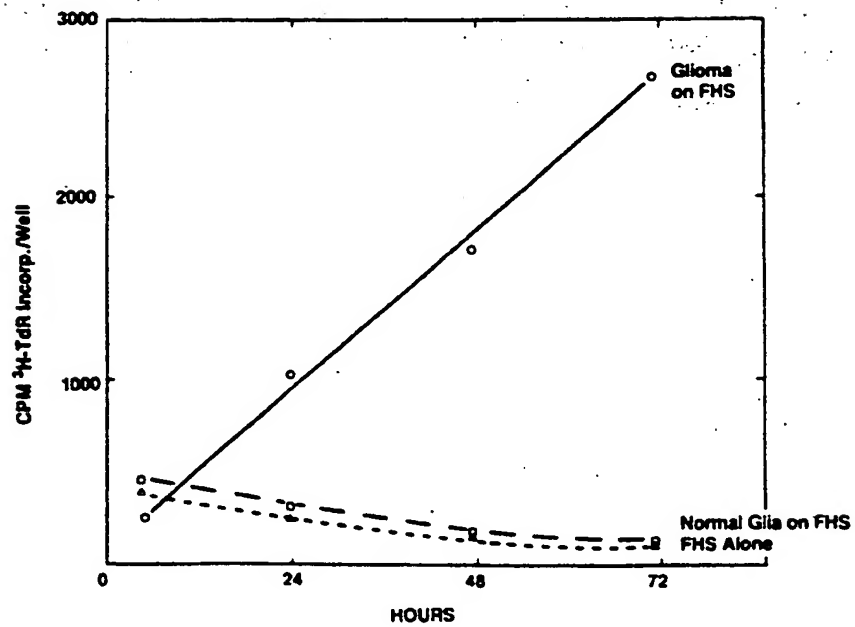


Figure 13.9. Legend on facing page.

If irradiation and trypsinization is carried out when the colony is about 100 cells in size, then the trypsinized cells will reclone. Three serial clonings may be performed within 6 wk by this method.

Other isolation techniques include: (1) distributing small coverslips or broken fragments of coverslips on the bottom of a petri dish. When plated out at the correct density, some colonies are found singly distributed on a piece of glass and may be transferred to a fresh dish or multiwell plate. (2) Capillary technique of Sanford et al. [1948]. A dilute cell suspension is drawn into a glass capillary tube (e.g., 50 μ l Drummond Microcap) allowing colonies to form inside the tube. The tube is then carefully broken on either side of a colony and transferred to a fresh plate. (3) Petri-perm dish. This is a petri dish with a thin gas-permeable base (see Chapter 8), which may be cut with scissors or a scalpel to isolate colonies. Since this means keeping the outside of the dish sterile, it needs to be handled aseptically and kept inside a larger sterile petri dish.

Suspension Clones

Outline

Draw colony into micropipette and transfer to a flask or the well of a multiwell plate.

Materials

24-well plates
medium
microcapillary pipettes
dissecting microscope
25-cm² culture flask

Protocol

Picking colonies is best done on a dissecting microscope.

1.

Pipette 1 ml of medium into each well of a 24-well plate

2.

Using a separate 50- μ l microcapillary pipette for each clone, place the tip of the pipette against the colony to be isolated and gently draw in the colony

3.

Transfer to a 24-well dish and flush out colony with medium. If from Methocel, the colony will settle, adhere, and grow out. If from agar, you

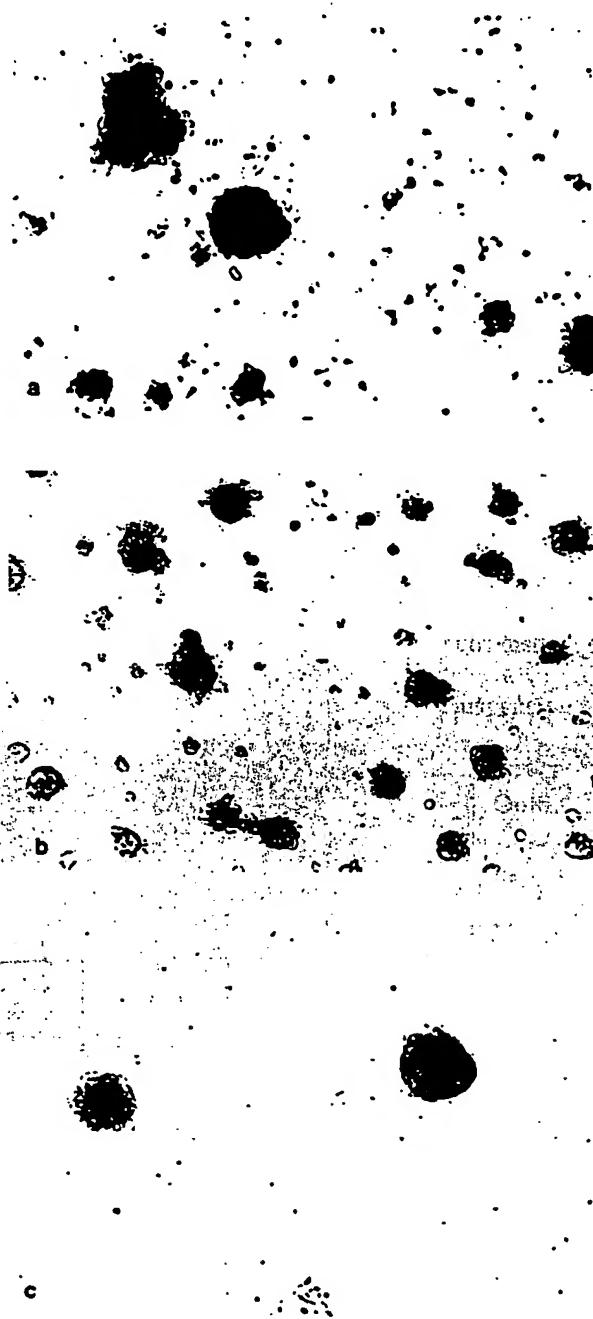


Fig. 13.10. Growth of melanoma, fibroblasts, and glia in suspension. Cells were plated out at 5×10^5 per 35-mm dish (2.5×10^5 cells/ml) in 1.5% methocel over a 1.25% agar underlay. Colonies were photographed after 3 wk. a. Melanoma. b. Human normal embryonic skin fibroblasts. c. Human normal adult glia. d. Colony-forming efficiency of normal and malignant glial cells in suspension. Unshaded bars, colonies of over eight cells (approximately), and stippled bars, colonies of over 32 cells (approximately). Colony counts were done on an Artek Colony Counter at different threshold settings.

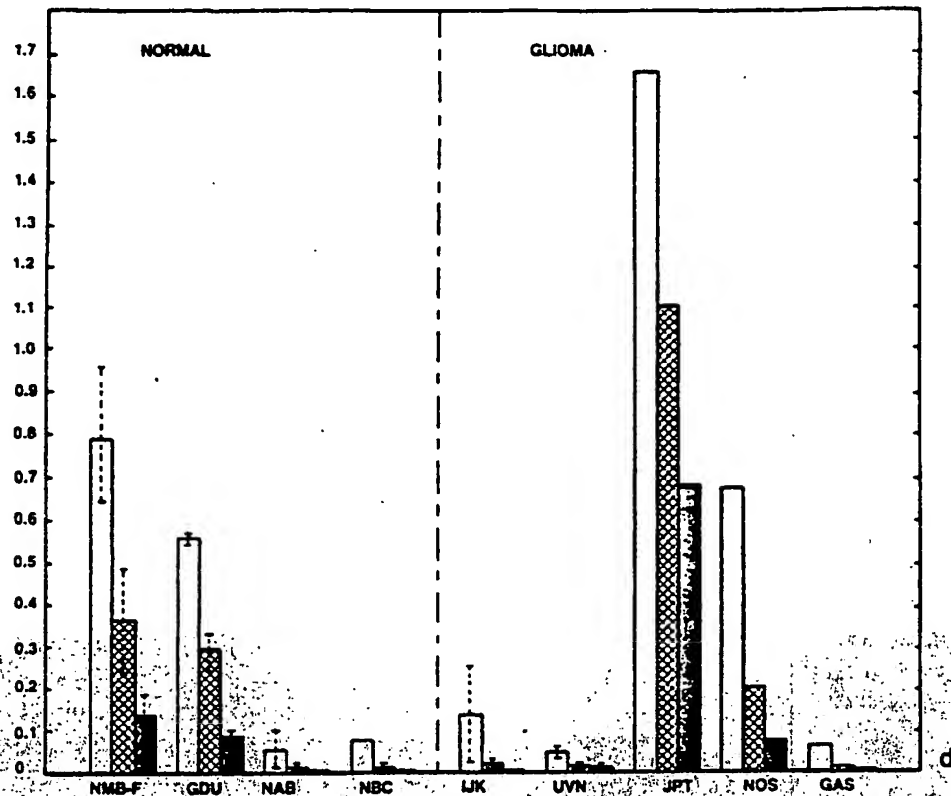


Figure 13.10. (continued)

may need to pipette the colony up and down a few times in the well to remove the agar

4.

Clones may also be seeded directly into a 25-cm² plastic flask standing on end (see above)

SELECTIVE MEDIA

Manipulating the culture conditions by using a selective medium is a standard method for selecting microorganisms. Its application to animal cells in culture is limited, however, by the basic metabolic similarities of most cells isolated from one animal in terms of their nutritional requirements. The problem is accentuated by the effect of serum, which tends to mask the selective properties of different media. Peehl and Ham [1980] were able to demonstrate that by using two different media, MCDB 105 and MCDB 151, with minimal amounts of dialyzed serum, either fibroblasts or epithelial cells could be grown preferentially from human foreskin.

Gilbert and Migon [1975, 1977] replaced the L-valine in the culture medium with D-valine and dem-

onstrated that cells possessing D-amino acid oxidase would grow preferentially. Kidney tubular epithelium and epithelial cells from fetal lung and umbilical cord may be selected this way.

Much of the effort in developing selective conditions has been aimed at suppressing fibroblastic overgrowth. Whei-Yang Kao [1977] used cis-OH-proline for this purpose, although this substance can prove toxic to other cells. Fry and Bridges [1979] found phenobarbitone inhibited fibroblastic overgrowth in cultures of hepatocytes and Braaten et al. [1974] were able to reduce the fibroblastic contamination of neonatal pancreas by treating the culture with sodium ethylmercurithiosalicylate. One of the more successful approaches was the development of a monoclonal antibody to the stromal cells of a human breast carcinoma [Edwards et al., 1980]. Used with complement, this antibody proved cytotoxic to fibroblasts from several tumors and helped to purify a number of malignant cell lines.

Selective media are also commonly used to isolate hybrid clones from somatic hybridization experiments. HAT medium, a combination of hypoxanthine, ami-

nopterin, and thymidine, selects hybrids with both hypoxanthine guanine phosphoribosyltransferase and thymidine kinase from parental cells deficient in one or the other enzyme (see Chapter 21) [Littlefield, 1964].

INTERACTION WITH SUBSTRATE

Selective Adhesion

Different cell types have different affinities for the culture substrate and will attach at different rates. If a primary cell suspension is seeded into one flask and transferred to a second after 30 min, a third after 1 hr, and so on, the most adhesive cells will be found in the first flask and the least adhesive in the last. Macrophages will tend to remain in the first flask, fibroblasts in the next few flasks, then epithelial, and finally hemopoietic cells in the last flask. Polinger [1970] used a similar procedure for the separation of embryonic heart muscle cells from fibroblasts.

If collagenase in complete medium is used for primary disaggregation of the tissue (see Chapter 11), most of the cells released will not attach within 48 hr unless the collagenase is removed. However, macrophages migrate out of the fragments of tissue and attach during this period and can be removed from other cells by transferring the disaggregate to a fresh flask after 48–72 hr-treatment with collagenase. This technique works well during disaggregation of biopsy specimens from human tumors.

Selective Detachment

Treatment of a heterogeneous monolayer with trypsin or collagenase will remove some cells more rapidly than others. Periodic brief exposure to trypsin removed fibroblasts from cultures of fetal human intestine [Owens et al., 1974] and skin [Milo et al., 1980], and Lasfargues [1973] found exposure of cultures of breast tissue to collagenase for a few days at a time removed fibroblasts and left the epithelial cells. EDTA, on the other hand, may release epithelial cells more readily than fibroblasts [Paul, 1975].

Dispase II (Boehringer, Mannheim) selectively dislodges sheets of epithelium from human cervical cultures grown on feeder layers of 3T3 cells (see below) without dislodging the 3T3 cells [Stanley, personal communication]. This technique may be effective in subculturing epithelial cells from other sources, excluding stromal fibroblasts.

Nature of Substrate

The hydrophilic nature of most culture substrates (see also Chapter 8) appears to be necessary for cell attachment, but little is known about variations in charge distribution on the cell surface and how different mosaic patterns may interact with different substrates. Since cell sorting in the embryo is a highly selective process and probably relates to differences in the distribution of charged molecules and specific receptor sites on the cell surface, qualitative and quantitative variations in substrate affinity should be anticipated in cultured cells. The relative infrequency

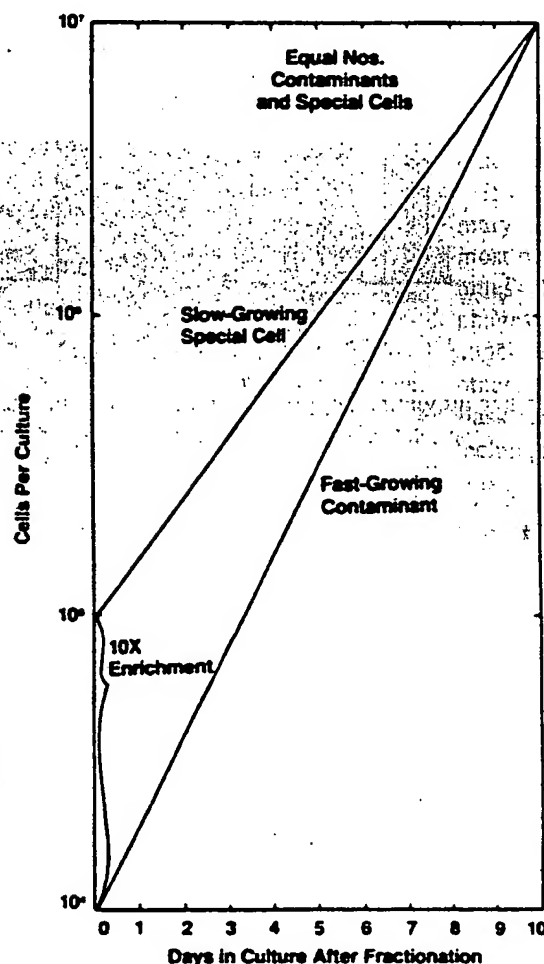


Fig. 13.11. Overgrowth of a slow-growing cell line by a rapidly growing contaminant. This is a hypothetical example, but it demonstrates that a 10% contamination with a cell population which doubles every 24 hr will reach equal proportions with a cell population which doubles every 36 hr after only 10 d growth.

with which this is actually found probably illustrates our ignorance of the subtlety of cell-cell and cell-substrate interactions.

The selective effect of substrates on growth may depend on both differential rates of attachment and growth, although in practice the two are indistinguishable. Polyacrylamide layers allow the cloning of tumor cells but not normal fibroblasts [Jones and Haskill, 1973, 1976]. Transformed cells proliferate on Teflon while most other cells will not [Parenjpe et al., 1975]. Macrophages will also attach to Teflon but do not proliferate. The dermal surface of freeze-dried pig skin was shown to allow growth of epidermal cells but not fibroblasts [Freeman et al., 1976]. Collagen, presumably the basis of the selection, has also been used in gel form to favor epithelial cell growth [Lillie et al., 1980] and in its denatured form to support endothelial outgrowth from aorta into a fibrin clot [Nicosia and Leighton, 1981].

Feeder layers. The conditioning of the substrate by feeder layers has been discussed already, in Chapter 8. Feeder layers can also be used for the selective growth of epidermal cells [Rheinwald and Green, 1975] and for repressing stromal overgrowth in cultures of breast (Fig. 13.8) and colon (see Fig. 20.1) carcinoma [Freshney et al., 1981]. The author has also been able to demonstrate that human glioma will grow on confluent feeder layers of normal glia while cells derived from normal brain will not [Freshney, 1980] (Fig. 13.9; see Chapter 16).

Semisolid supports. Transformation of many fibroblast cultures reduces anchorage dependence of cell proliferation (see Chapter 16) [Macpherson and Mon-

tagnier, 1964]. By culturing the cells in agar (see above) after viral transformation, it is possible to isolate colonies of transformed cells and exclude most of the normal cells. Most normal cells will not form colonies in suspension with the same high efficiency as virally transformed cells, although they will often do so with low plating efficiencies. Colonies of hemopoietic cells will form in semisolid media [Metcalf, 1970], but these usually mature to nondividing differentiated cells and cannot be subcultured (see also Chapter 16). The difference between virally transformed fibroblasts and untransformed cells is not seen as clearly in attempts at selective culture of spontaneously arising tumors. Experiments in the author's laboratory have shown that normal glia and fetal skin fibroblasts will form colonies in suspension just as readily as glioma and melanoma (Fig. 13.10).

Cell cloning and the use of selective conditions have a significant advantage over physical cell separation techniques (next chapter) in that contaminating cells are either eliminated entirely by clonal selection or repressed by constant or repeated application of selective conditions. Even the best physical cell separation techniques will still allow some overlap between cell populations such that overgrowth will recur. As long as this situation exists, a steady state cannot be achieved and the constitution of the culture is altering continuously. From Figure 13.11 it can be seen that a 90% pure culture of line A will be 50% overgrown by a 10% contamination with line B in 10 days, given that B grows 50% faster than A. For continued culture, therefore, selective conditions are required in addition to, or in place of, physical separation techniques.

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